Verocytotoxin Expression in *Escherichia coli*

by

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NUMEROUS ORIGINALS
IN COLOUR

DEDICATION

I dedicate this thesis to my parents
for all their love and support over the years

Declaration

The work contained in this thesis is the result of original research by myself under the supervision of Dr C S Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

Sian L Fox
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ABBREVIATIONS

A	Absorbance
A/E	Attaching and effacing
AMPS	Ammonium persulphate
Ap	Ampicillin
bp	Base pair
BSA	Bovine serum albumin
BME	β-mercaptoethanol
BRL	Boehringer-Mannheim
BRL	Bethesda Research Laboratories
^{o}C	Degrees celsius
CFU	Colony forming units
cm	Centimetre
Corp	Corporation
CLDT	Cytolethal distending toxin
Cm	Chloramphenicol
CsCl	Caesium chloride
DA	Diffuse adherence
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dUTP	2'-deoxyuracil 5-triphosphate
DDW	Double distilled water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide

ED	Oedema disease
EDP	Oedema disease principle
EDTA	Diaminoethane tetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FLB	Formaldehyde loading buffer
FVT	Faecal verotoxin
xg	Gravitational force
g	Gramme
HC	Haemorrhagic colitis
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGMF	Hydrophobic grid membrane filter
HUS	Haemolytic uraemic syndrome
IPTG	β-D-thiogalactopyranoside
Kn	Kanamycin sulphate
Kb	Kilobase
KDa	Kilodalton
KV	Kilovolt
LA	Localised adherence
LPS	Lipopolysaccharide
LT	Heat labile enterotoxin
M	Molar
mA	Milliamp
MAb	Monoclonal antibody
MDa	Megadalton
μF	Microfarads

μg	Microgramme
mg	Milligramme
min	Minute
mM	Millimolar
MOPS	3-[N-Morpholino propane sulfonic acid]
MW	Relative molecular weight
NAb	Neutralising antibody
NBL	Northumbria Biologicals Limited
ng	Nanogrammes
NSF	Non-sorbitol fermenter
nm	Nanometre
OD	Optical density
PAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
%	Percentage
PFU	Plaque forming units
pg	Picogrammes
PhoA	Alkaline phosphatase
RIP	Ribosome inhibiting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
sec	Second
ShT	Shiga toxin

Sigma 104	p-nitrophenyl phosphate disodium salt
Slt	Shiga-like toxin
ST	Heat stable toxin
Tc	Tetracycline hydrochloride
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylaminomethane
Tn	Transposon
Tris	Tris-hydroxymethylaminomethane
TTP	Thrombotic thrombocytopenic purpura
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet radiation
VRC	Vanadyl ribonucleoside complex
V	Volt
VT	Verotoxin
VTEC	Verotoxin producing *E. coli*
v/v	Volume by volume
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indoxyl β-D-galactoside
XP	5-Bromo-4-chloro-3-indolyl β-D-phosphate

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SUMMARY

In order to examine the physiology of verotoxin (VT) production *in vitro*, it was necessary to develop an assay to enable specific measurement of VT1 and VT2 expression. Initial efforts focussed on quantifying VT1 and VT2 messenger RNA synthesis. RNA extraction and electrophoresis was consistently achieved, however, subsequent probing of Northern filters was not successful. A gene fusion between the *slt*-II operon and the transposon vector Tn*phoA*, such that expression of the *phoA* gene was brought under the control of the *slt*-II promoter, was then developed. The presence and site of insertion of Tn*phoA* within the *slt*-II operon was confirmed by restriction and sequence analysis, and a single copy fusion derivative within the VT2-producing *E. coli* strain E32511 was then obtained by exchange recombination. Use of the resulting single copy fusion derivative (E32511 SLF22/1) and a plasmid encoded *slt*-II::Tn*phoA* fusion (pSC105) demonstrated significant differences in the synthesis, secretion and localisation abilities of VT1 and VT2 during growth under aerobic, anaerobic and iron limiting conditions. These characteristics could have important implications on the relative abilities of the two toxins to cause disease *in vivo*.

CHAPTER ONE

1 Introduction

1.1 General introduction and historical background

Verocytotoxin producing *Escherichia coli* (VTEC) were first recognised as etiological agents of diarrhoea by Konowalchuk *et al.* in 1977. While investigating the usefulness of Vero (African green monkey kidney) cells for detecting cytotonic *E. coli* heat labile enterotoxin (LT), they found a cytotoxin in the culture filtrates of a number of *E. coli* strains. This toxin, designated verocytotoxin (VT) was cytotoxic for Vero but not for Y1 (mouse adrenal) or CHO (Chinese hamster ovary) cells. In addition it was labile to heat and not neutralised by antisera to LT. Shortly after it became apparent, by neutralisation tests, that there was more than one type of cytotoxin affecting Vero cells (Konowalchuk *et al.*, 1978). Subsequently it was shown that the genes encoding toxin production were bacteriophage mediated (Scotland *et al.*, 1983a; Williams Smith *et al.*, 1983), and in earlier work by O'Brien *et al.* (1982) that the VT from Konowalchuk's reference strain, H30, was very closely related both biologically and immunologically to shiga toxin (ShT) produced by strains of *Shigella dysenteriae* type 1.

Since the initial description of VT by Konowalchuk and his colleagues, numerous studies have demonstrated the pathogenicity of VTEC. Both Konowalchuk *et al.* (1977) and Scotland *et al.* (1980) documented strains producing VT (VT^+) among traditional serogroups of enteropathogenic *E. coli* (EPEC). Wade *et al.* (1979) in England first noted the presence of cytotoxin-producing *E. coli* 026 strains, (especially flagellar type H11), in association with bloody diarrhoea. This finding was supported by reports from Scotland *et al.* (1979) in the UK and Wilson and Bettleheim (1980) in New Zealand. However, the major breakthroughs occurred in 1983 with the publication of studies that linked strains of *E. coli* producing high levels of VT, to two life threatening conditions of previously unknown cause, haemorrhagic colitis (HC), (Riley *et al.*, 1983; Johnson *et al.*, 1983) and the haemolytic uraemic syndrome (HUS), (Karmali *et al.*, 1983). Riley *et al.*

(1983) and Johnson *et al.* (1983) both associated the outbreaks and sporadic cases of HC they documented with *E. coli* O157:H7, a serotype not previously recognised as a cause of diarrhoeal disease in humans. The discovery that VTEC are epidemiologically associated with HC and HUS generated tremendous interest in these organisms and their toxins. A major area of multidisciplinary research interest in VTEC has resulted in significant progress in understanding the clinical and epidemiological features, natural history, laboratory diagnosis and pathogenesis of VTEC infections in humans and other animals, as well as fundamental knowledge of the structure-function relationship and molecular biology of VT's.

1.2 Nomenclature

Nomenclature for this group of cytotoxins remains confusing. While they were initially called verotoxins (VT's), the discovery by O'Brien *et al.* (1982) that certain VT's were neutralised by polyclonal antisera raised against ShT from *S. dysenteriae* type 1 led to the development of two different nomenclatures now used for the *E. coli* Vero cell cytotoxins, VT's and shiga-like toxins (SLT's). The terms VT and SLT are synonymous and it would be far better and less confusing if a uniform nomenclature were adopted. Until recently only two distinct VT's were recognised, verotoxin 1 (VT1) and verotoxin 2 (VT2). A third VT was subsequently found in pigs with oedema disease (VTe). The recent identification of variants of both VT2 (VT2v's), (Ito *et al.*, 1990; Schmitt *et al.*, 1991) and VTe (VTeva), (Gannon *et al.*, 1990) has only added to this confusion. The VT designation seems better suited to address the present and possible future variants, because it places VTe into a separate group. This would avoid confusion when referring to the VT2 variants and the VTe variants. The abbreviations, shown in table 1.1, will therefore be utilised throughout this thesis.

Table 1.1 The family of Verocytotoxins

Toxin	Operon	Reference
Class I		
VT1	*slt*-I	O'Brien & LaVeck (1983); Newland *et al.* (1985); Huang *et al.* (1986); Jackson *et al.* (1987a); Calderwood *et al.* (1987); De Grandis *et al.* (1987); Noda *et al.* (1987); Petric *et al.* (1987b).
Class II		
VT2	*slt*-II	Jackson *et al.* (1987b); Newland *et al.* (1987); Yutsudo *et al.* (1987); Downes *et al.* (1988); Dickie *et al.* (1989).
VTe	*slt*-IIv	Gyles *et al.* (1988); Weinstein *et al.* (1988a); Macleod & Gyles (1990).
VT2 variants		
VT2va	*vtx*2ha	Oku *et al.* (1989); Ito *et al.* (1990).
VT2vb	*vtx*2hb	Oku *et al.* (1989); Ito *et al.* (1990).
VT2vc	*slt*-IIc	Schmitt *et al.* (1991).
VTe variants		
VTeva	*slt*-IIva	Gannon *et al.* (1990).

1.3 Characterisation, structure and function of VT's

1.3.1 VT classes I and II - biological activity and immunological relationships

Konowalchuk *et al.* (1977, 1978) first described at least three distinct *E. coli* VT's, two of them being from human strains and a third from a porcine strain. Emerging evidence has since indicated that VT's are a family of structurally and functionally related cytotoxins. They can be divided into classes I (VT1) and II (VT2, VTe, VT2 and VTe variants) and can be distinguished serologically or on the basis of gene sequence or host specificity. The members of the two classes discovered to date are shown in table 1.1.

Class I : The first *E. coli* cytotoxin to be discovered, VT1, from strain H30 (Konowalchuk *et al.*, 1977, 1978), was shown to be immunologically very similar to ShT of *S. dysenteriae* type 1 (O'Brien & LaVeck, 1982). The isoelectric point (pI) (table 1.2), subunit structure and biological activities were also similar with both toxins showing complete cross-neutralisation (O'Brien & LaVeck, 1983).

Class II : Class II encompasses an ever growing family of closely related VT's. VT2 possesses the same biological activities as VT1, both are toxic to the same cell lines, cause paralysis and death in mice and fluid accumulation in rabbit ileal loops (Strockbine *et al.*, 1986). VT2 however, is antigenically distinct. It is not neutralised by polyclonal antiserum to ShT and VT1 and conversely, VT2 neutralising antibodies do not neutralise ShT or VT1 (Strockbine *et al.*, 1988). VTe, also known as "oedema disease principle" or EDP, (Lingwood & Thompson, 1987), has been found in pigs with oedema disease (ED). ED attacks thriving pigs about one week after weaning, the major feature being progressive incoordination leading to partial or complete paralysis and death. The disease is associated with bowel colonisation by specific *E. coli* serogroups, notably, O138, O139 and O141 which produce VTe. VTe is antigenically similar to VT2 being neutralisable with antiserum to VT2 but not to VT1 (Marques *et al.*, 1987). It is more heat-labile however, than VT1 and VT2. Also, whereas VT1 and VT2 possess the same biological activities, VTe is only cytotoxic to Vero cells and is non-cytotoxic to HeLa cells (Marques *et al.*, 1987). This difference in biological activity on tissue culture cells has been one of the criteria commonly used to differentiate between VT2 and VTe *in vitro*. More recently however, toxins displaying similar biological properties to VTe have been associated with human disease as well (section 1.4.3.4), (Oku *et al.*, 1989; Gannon *et al.*, 1990; Schmitt *et al.*, 1991). These toxins can be distinguished on the basis of gene sequence and have been termed the VT2 (Ito *et al.*, 1990; Schmitt *et al.*, 1991) and VTe variants (Gannon *et al.* 1990).

Table 1.2 Comparison of the processed ShT/VT1, VT2 and VTe subunits

Subunit	Base Pairs	Amino acids	Molecular weight	pI
A				
ShT/VT1[a]	879	296	32,225/32,211	11.1
VT2	888	293	33,135	9.8
VTe	891	297	33,050	8.7
B				
ShT/VT1	207	69	7,690	5.9
VT2	210	70	7,817	5.4
VTe	204	68	7,565	10.2

[a] The ShT and VT1 A subunits have one amino acid difference, a threonine at position 45 in ShT and a serine at the corresponding position in VT1. This is reflected by a slight difference in the molecular weights of the A subunits.

1.3.2 Subunit structure

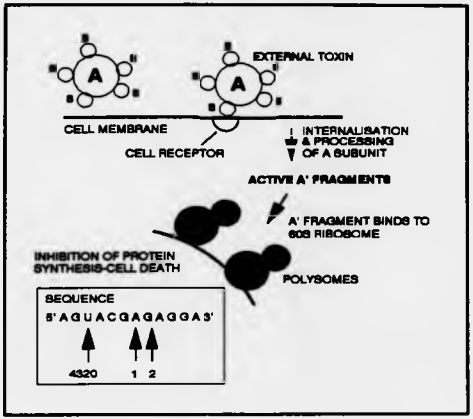
VTs are subunit toxins which like ShT holotoxin (Donohue-Rolfe *et al.*, 1984), consist of one enzymatically active A subunit non-covalently linked to multiple copies, most likely five, of a B subunit (O'Brien & Holmes, 1987). A comparison of the processed subunits are shown in table 1.2. VT1 is almost identical to ShT (Noda *et al.*, 1987) and the holotoxin is thought to have a relative molecular weight (MW) of 70 Kilodaltons (KDa). Estimated MW for the toxins have varied however. MW determined by gel filtration chromatography have been reported of 32 and 45 KDa (O'Brien & LaVeck, 1983), 25 to 39 KDa (O'Brien & LaVeck, 1983; Petric *et al.*, 1987b) and 42 KDa (Head *et al.*, 1988a) for ShT, VT1 and VT2 holotoxins respectively. The MW for VTe holotoxin was also much lower (40 to 43 KDa) than that expected from a 1-A-subunit-5-B-subunit toxin molecule (MW 70,984), (MacLeod *et al.*, 1991). Binding of the toxin to

the gel matrix could be an explanation for the MW values determined by gel filtration. Padhye *et al.* (1986) isolated a cytotoxin from *E. coli* O157:H7 strain 932 which was not neutralisable with antisera to ShT, had a pI of 5.2, and therefore resembled VT2. This toxin differed from the class I and class II VT's however, in that it appeared to lack the characteristic A and B subunit structure and migrated instead as a single 64 KDa band when subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Studies of its biological effects in mice suggested that this toxin may be responsible for colonic mucosal haemorrhage and lymphoid tissue lesions (Padhye *et al.*, 1987).

1.3.3 Mode of action

It has been shown (Endo *et al.*, 1988; Saxena *et al.*, 1989) that the toxic action of ShT, class I and class II VT's is the same in sensitive mammalian cells (figure 1.1). The A subunit is enzymatically active and like the A chain of ShT (Reisbig *et al.*, 1981) is responsible for inhibiting protein synthesis through the catalytic inactivation of 60 S ribosomal subunits (section 1.3.3.2). The B subunit on the other hand, mediates receptor binding, cytotoxic specificity and extracellular localisation of the holotoxin (section 1.3.3.1), (Jackson *et al.*, 1990). The 32 KDa A subunit can be cleaved *in vitro* into a catalytically active A1 fragment of 27 KDa and an A2 fragment of 4.5 KDa by reduction and treatment with trypsin or endogenous proteases. Treatment of intact toxin with trypsin in the absence of reducing agents however, results in the A2 fragment remaining linked to the A1 fragment by a disulphide bond. Whereas nicking and reduction of intact ShT results in increased activity in a cell-free system and reduced toxic activity on intact cells (Reisbig *et al.*, 1981), nicking in the A subunit of VT1 has been found not to affect the biological or physical properties of the toxin, such as cytotoxic activity, heat stability and time necessary for binding to Vero cells (Kongmuang *et al.*, 1988).

Figure 1.1 Toxic action of Shiga toxin and the Verotoxin family



The B subunit binds to specific receptors on the surface of the cell and the A subunit internalised and proteolytically cleaved to an A' fragment. The latter has N-glycosidase activity and inactivates 60 S ribosomal subunits by cleaving the N-glycosidic bond at nucleotide residue 4324 in 28 S ribosomal RNA. This inhibits protein synthesis and leads to cell death. The site of action is the same as that of Ricin, a potent cytotoxic protein produced by the castor bean (*Ricinus communis*).

1 - acting site for Ricin, Shiga toxin and Verotoxin.
2 - acting site for the fugal toxin α-Sarcin (Endo & Wool, 1982).

1.3.3.1 The role of the B subunit

As mentioned above (section 1.3.1), VT1 and VT2 are cytotoxic for both Vero and HeLa cells, whereas VTe and VT2v's are cytotoxic for Vero and not HeLa cells. Construction of hybrid cytotoxins comprised of the individual subunits of ShT, VT1, VT2 and VTe has demonstrated that the different cell specificity of VTe compared with ShT, VT1 and VT2 is dictated by the VTe B subunit (Weinstein *et al.*, 1989). The B subunit of ShT, VT1 and VT2 has been shown to bind to cell surface receptors containing terminal galactose-α(1-4)-galactose disaccharides and with high affinity to the glycolipid, globotriosyl ceramide (Gb_3), (Lindberg *et al.*, 1987; Lingwood *et al.*, 1987; Waddell *et al.*, 1988). In contrast, VTe binds minimally to Gb_3 and binds with high affinity to globotetraosyl ceramide (Gb_4), which contains an additional N-acetylgalactosamine (GalNAc) residue in β1-3 linkage to which VT1 and VT2 do not bind (De Grandis *et al.*, 1989). A recent study has compared the glycolipid receptor specificities of VT2, VTe and VT2va (table 1.3), (Samuel *et al.*, 1990). It was found that both VTe and VT2va recognise Gb_3 contained in both Vero and HeLa cells and furthermore, VT2va (Oku *et al.*, 1989) did not bind Gb_4. It has therefore been suggested that the effects of ShT and VT's in humans and animal models reflects the relative toxin binding affinity as well as the differential tissue distribution, cell surface concentration of receptors and sensitivity of cells to toxin (Boyd & Lingwood, 1989; Samuel *et al.*, 1990). One model for this dependance proposes that when toxin is bound to a cell, all or at least several B subunits of the holotoxin must bind to separate receptor molecules. This in turn, may cause local destabilisation of the membrane and allow the enzymatically active A subunit to translocate the membrane to the cytoplasm. Receptors must therefore occur at a sufficient density and be attracted by sufficient avidity to create this membrane disturbance.

Table 1.3 Structures of glycolipids that bind VT2, VTe and VT2 variants (Samuel et al., 1990)

Name	Structure[a]	Binding[b]			Source
		VTe	VT2v[d]	VT2	cells
Galabiosylceramide (Gb_2)	Galα1-4Galβ1-1Cer	-	+	+	HeLa
Globotriaosylceramide (Gb_3)	Galα1-4Galβ1-4Glcβ1-1Cer	+	+	+	HeLa, PSI[c], Vero
Globotetraosylceramide (Gb_4)	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	+	-	(+)	Vero, PSI[c]
Galactosylglobotetraosylceramide (Gb_5)	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	+	-	-	Vero

[a] The underlined sugars are the consensus binding sequence.

[b] Binding of glycolipids on thin-layer chromatograms was graded as follows; +, strong binding; (+), weak binding; -, no binding.

[c] Piglet small intestine mucosal cells.

[d] VT2v corresponds to VT2va (Ito et al. 1990) which is antigenically indistinguishable to VT2vb (Ito et al., 1991).

In addition to cell specificity, it has been shown that the B subunit also dictates localisation of the toxin within the bacterial cell (Weinstein *et al.*, 1989). Whereas VTe is predominantly extracellular, VT1 is almost completely cell-associated and VT2 more equally distributed between the cell-associated and extracellular fraction (Strockbine *et al.*, 1986). The mechanisms by which the toxins are distributed according to their B subunit have not been explored but the carboxyl terminus is thought to be involved in determining the extracellular localisation of VTe (Jackson *et al.*, 1990).

1.3.3.2 The role of the A subunit

The process of internalisation of the A subunit into the cytosol has not been precisely established but it is thought that like ShT, entry requires a Ca^{2+} flux through naturally occurring calcium channels (Sandvig & Brown, 1987). This hypothesis is supported by the fact that Verapamil, a licenced calcium channel blocker, prevents cell bound A subunit from entering Vero cells. It is not known at what point during entry that the disulphide bond of the protein toxin is reduced.

The enzymatic activity of ShT and VT's is the same as that of Ricin, a potent cytotoxic protein produced by the castor bean - *Ricinus communis* (Endo *et al.*, 1987, 1988; Igarashi *et al.*, 1987), (figure 1.1). They are N-glycosidases that inactivate 60 S ribosomal subunits by cleaving the N-glycosidic bond at adenine 4324 in 28 S ribosomal ribonucleic acid (rRNA). Subsequent treatment of the RNA with aniline causes release of a distinctive fragment of 400 nucleotides from 28 S rRNA (Endo *et al.*, 1988). This specific N-glycosidase activity is not nucleotide sequence specific because the target sequence (figure 1.1), which is conserved between eukaryotes and prokaryotes, is only modified when this sequence is part of the eukaryotic ribosome (Endo *et al.*, 1987). For ShT it has been shown that the enzymatic activity of the A1 fragment can only be expressed when the 60 S subunit is in the 80 S complex (Reisbig *et al.*, 1981). In contrast the A chain of Ricin can inactivate the isolated 60 S subunit. The mode of inhibitory

action of all toxins involves the blocking of elongation-factor-1-dependent binding of aminoacyl-transfer RNA to ribosomes (Igarashi *et al.*, 1987; Endo *et al.*, 1988). This may explain why most VTEC infections have an initial watery diarrhoea, due to inhibition of salt and water absorption by the enterocytes on the villus tips.

1.4 Genetic basis and regulation of VT's

1.4.1 Toxin-converting bacteriophages

Production of VT1 and VT2 has been shown to result from infection by lysogenic bacteriophage carrying the structural genes for toxin production (Scotland *et al.*, 1983a; Williams Smith *et al.*, 1983). This was first demonstrated by Scotland *et al.* (1980) who showed that the property could be transferred from a strain of *E. coli* O26:H11, strain H19, to *E. coli* K12. A family of VT-converting phages has been found to exist in nature (O'Brien *et al.*, 1984). The VT phages from strain H19 (H19A and H19B) and phage 933J from O157:H7 strain 933 have very similar morphology when examined by electron microscopy. Comparison of phage-encoded polypeptides and restriction enzyme analysis of phage deoxyribonucleic acid (DNA) also indicated close relatedness (O'Brien *et al.*, 1984). Subsequently, comparison of a limited number of VT-encoding phages (Willshaw *et al.*, 1987) indicated that a VT1 encoding phage from *E. coli* O157:H7 strain E30480 (a VT1 and VT2 producer) and a VT2 encoding phage from O157:H$^-$ strain E32511 were closely related, being morphologically indistinguishable and having similar DNA restriction digest patterns. VT2 encoding phage 933W, from the O157 strain 933 (Strockbine *et al.*, 1986) was also found to belong to this family of O157 phages determining VT production. However, no VT2 encoding phage from strain E30480 was found in this study. Later experiments showed that the VT2 encoding phage was only released after ultra violet (UV) induction of E30480. This phage remains to be characterised. In addition to being morphologically distinct, the molecular size of the phage DNA and restriction enzyme digests of the VT-encoding

phages from the O157 strains were clearly distinguishable from the VT1 encoding phage of *E. coli* O26 strain H19. There has been no report of a single phage carrying more than one VT operon.

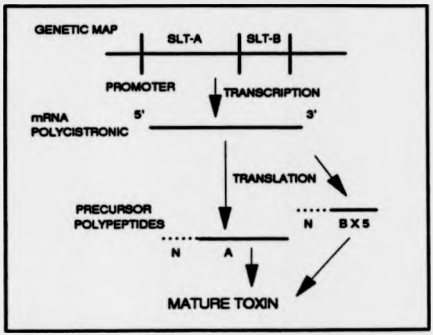
No phage mediated cytotoxin production has been demonstrated for *S. dysenteriae* type 1 or other *Shigella* species tested (Weinstein *et al.*, 1988a) and phage mediated production of VTe has not been shown (Williams Smith *et al.*, 1983). Some VT2 producing *E. coli* strains have also been documented as not harbouring toxin-converting phage (Marques *et al.*, 1987). It is possible that these strains are lysogenised with unstable or defective phage or that the conditions used for induction or detection of phage were not optimum. Another explanation is that the toxin structural genes are located on the chromosome or on plasmids of these strains. Although no plasmid involvement has been demonstrated, transfer of genes coding for VTe from a field isolate to a K12 strain by conjugation has been reported (Williams Smith *et al.*, 1983).

These phages represent potentially mobile elements within the gene pools of *E. coli* and related organisms, as transduction could serve to disseminate the toxin structural genes among related bacteria. Indeed other strains of bacteria including non-enterohaemorrhagic *E. coli*, enterohaemorrhagic *E. coli* (EHEC), *Shigellae*, *Vibrios* and *Campylobacter* produce VT's in small amounts (O'Brien & Holmes, 1987). It is not known whether low-level VT producing organisms carry genes homologous to the VT genes carried by VTEC and differ only with respect to the regulation of their expression. This widespread production might suggest that VT's may also function in bacterial cell metabolism and are not necessarily virulence factors alone.

1.4.2 Operon structure

The structural genes for class I and class II VTs have been cloned and the complete nucleotide sequence of the structural genes for the A and B polypeptides and the adjacent regulatory sequences determined (table 1.1, section 1.4.3). The structural genes for each VT are organised into an operon (figure 1.2) which is transcribed from a promoter upstream of the A subunit gene (Sung *et al.*, 1990). Jackson *et al.* (1987a) identified a potential promoter located 5' to the *slt*-I B subunit gene which evidence suggests can function in the absence of the promoter located 5' to the *slt*-I A subunit gene (Newland *et al.*, 1985; Huang *et al.*, 1986; Weinstein *et al.*, 1988b). It remains to be seen however, whether the promoter functions in a normal chromosomally situated *slt*-I operon. No evidence of an independent promoter for the B subunit gene of the *slt*-II operon has been found (Sung *et al.*, 1990). An untranslated intergenic space of 12, 14 and 15 nucleotides separates the A and B subunit genes in the *slt*-I, *slt*-II and *slt*-IIv operons respectively (Jackson *et al.*, 1987b; Gyles *et al.*, 1988; Weinstein *et al.*, 1988a). This results in translation of the A and B subunit genes of the *slt*-I and *slt*-IIv operons in the same reading frame, while the A and B subunit genes of the *slt*-II operon are translated in different reading frames. The existence of separate ribosome binding sites 5' to the A subunit gene and in the untranslated space between the subunit coding sequences suggests that the A and B subunit genes are translated independently from a polycistronic messenger RNA (mRNA), (Jackson *et al.*, 1987b). It is thought that enhanced translation of the B subunit coding sequence from the latter results in the holotoxin consisting of multiple copies of the B subunit for every A subunit. Presumed *E. coli* signal peptidase I cleavage sites (Oliver, 1985) have been identified for the A and B polypeptides and analysis of the translated amino acid sequences indicate that the A and B polypeptides are synthesised as precursors with hydrophobic signal sequences at the amino termini that are removed by proteolysis to form the mature A and B polypeptides. Whereas processing of the A and B subunits of VT1 has been observed (Newland *et al.*, 1985), comparison between the sizes of the VT2 subunits from whole

Figure 1.2 Organisation and expression of the Verotoxin operon



The structural genes for VT (*slt*A and *slt*B) in *Escherichia coli* appear to be transcribed as an operon (*slt*), from a promoter upstream of *slt*A. It is likely however, that the A and B subunits are translated independently as analysis of translated amino acid sequences indicate that the A and B polypeptides contain hydrophobic signal sequences at the amino termini and therefore are probably synthesised as precursor forms of secreted periplasmic proteins.

cell lysates and polymyxin B extracts failed to detect evidence of the processing of the VT2 subunits (Newland *et al.*, 1987). It is unclear whether only the mature, or only the precursor forms of the subunits are being detected.

1.4.3 Gene cloning and DNA sequence homologies

1.4.3.1 VT1

The structural genes for VT1, designated *slt*-I A and *slt*-I B were cloned and sequenced from the genomes of bacteriophage 933J (Newland *et al.*, 1985; Jackson *et al.*, 1987a) and H19 (Willshaw *et al.*, 1985; Huang *et al.*, 1986; Calderwood *et al.*, 1987). The ShT locus of *S. dysenteriae* 1 is almost identical to that of VT1, being greater than 99% homologous (Strockbine *et al.*, 1988). There are only three nucleotide differences in the A subunit resulting in a single amino acid substitution, whereas the B subunits are identical. VT1 and ShT have identical signal peptides for the A and B subunits, as well as identical ribosome binding sites. Nucleotide sequence similarities upstream of the start sites of the A subunits suggest that both toxins have homologous promoter and regulatory regions.

1.4.3.2 VT2

The structural genes for VT2, *slt*-II A and *slt*-II B from phage 933W have also been cloned (Newland *et al.*, 1987; Jackson *et al.*, 1987b). Comparison of the VT1 and VT2 nucleotide sequences indicates that they share 58% overall nucleotide and 56% amino acid sequence homology (table 1.4), (Jackson *et al.*, 1987b). The sequence similarities are greatest in the areas coding for the mature B subunit and the processed A1 polypeptide.

Table 1.4 **Nucleotide and deduced amino acid sequence homologies of the processed *slt*-I : *slt*-II and *slt*-II : *slt*-IIv subunits**

	A subunit		B subunit		A subunit		B subunit	
	slt-I	*slt*-II	*slt*-I	*slt*-II	*slt*-II	*slt*-IIv	*slt*-II	*slt*-IIv
Nucleotide seq. homology (%)	57		60		94		80	
Amino acid seq. homology (%)	55		57		94		84	

VT2 has consistently been reported not to be cross-neutralised by either polyclonal antibody (PAb) or monoclonal antibody (MAb) to ShT or VT1. The lack of cross-reactivity between ShT/VT1 and VT2 is puzzling however, since the three toxins have the same specific mechanism of action and apparently bind to the same specific sugar structure, suggesting the potential presence of very similar epitopes in all three toxins. In one study (Donohue Rolfe *et al.*, 1989), three of four MAbs produced, demonstrated that ShT and VT2 were related. The reason why this group obtained cross-reactive MAbs when a number of other investigators failed to do so is uncertain but may be due to use of a toxin for immunisation that had been purified to a high degree with a novel affinity column based on the sugar-binding specificity of the B subunit. It is possible that by doing this they were able to free the preparation of contaminants that inhibit the production of cross-reactive antibody. It is also possible that the simple single-step purification method used in this study resulted in less damage to the protein and that the immunogenicity of cross-reactive epitopes was maintained. There was however, no evidence to support these speculations.

1.4.3.3 VTe

The structural genes for VTe, *slt*-IIvA and *slt*-IIvB have been cloned from the total cellular DNA of ED-causing *E. coli* strains (Gyles *et al.*, 1988; Weinstein *et al.*, 1988a). Comparison of the genes determining the putative processed A and B subunits of VT2 and VTe indicates that they are very closely related (table 1.4), (Gyles *et al.*, 1988). The toxin genes exhibit a very high degree of sequence similarity in the upstream sequences (suggesting a common regulatory mechanism), the signal peptide and the A2 fragment region, suggesting an extremely close evolutionary relationship. Despite the high degree of homology however, the pI's of the B subunits of these toxins are very different due to significant variation of individual amino acids at comparable positions in the B polypeptides of VTe and VT2. Also, the greatest amino acid sequence differences were found near the carboxy termini of the respective B subunits and may explain through binding differences the different spectrum of cytotoxicity observed for the two toxins.

1.4.3.4 VT2 variant toxins (human and porcine)

A VT purified from an *E. coli* O91:H21 strain (B2F1) isolated from a patient with HUS was found to have greatly reduced activity in HeLa cells (Oku *et al.*, 1989). The initial characterisation of this strain led to the speculation that the VT being produced was VTe. The subsequent cloning and sequencing of genes from *E. coli* O91:H21 identified two distinct genes capable of producing a VT similar to VT2 both of which had RNA N-glycosidase activity (Ito *et al.*, 1990). The nucleotide sequences of the two operons designated *vtx*2ha and *vtx*2hb were determined and found to be nearly identical (table 1.5). Both encoded A and B subunits of 319 and 89 amino acids respectively, the A and B genes being separated by a stretch of 14 nucleotides. It was speculated that the presence of two nearly identical *vtx*2h operons in cellular DNA can be explained by a mechanism such as insertion sequence-mediated gene duplication and Ito and co-workers are currently investigating whether the *vtx*2h operons are flanked by insertion

sequence-like elements. Nucleotide and deduced amino acid sequence homologies of the *vtx*2ha genes with those of the *vtx*2hb, *slt*-II and *slt*-IIv genes are shown in table 1.5.

Table 1.5 **Nucleotide and deduced amino acid sequence homologies of the *vtx*2ha genes with those of the *vtx*2hb, slt-II and slt-IIv genes** (Ito *et al.*, 1990)

	Nucleotide sequence homology[a] (%)		Deduced amino acid sequence homology[b](%)	
Operons	**A subunit**	**B subunit**	**A subunit**	**B subunit**
*vtx*2ha	100 (100)[c]	100 (100)	100 (100)	100 (100)
*vtx*2hb	99.3 (99.1)	98.9 (98.6)	99.4 (99.3)	98.9 (100)
slt-II	98.6 (98.5)	95.5 (94.8)	98.7 (98.3)	96.6 (97.1)
slt-IIv	94.5 (94.5)	82.8 (78.1)	94.4 (96.6)	85.4 (82.9)

[a] Number of bases of *vtx*2ha genes are used as common denominators.

[b] Number of amino acid residues of *vtx*2ha gene products are used as common denominators.

[c] Values in brackets are for the products obtained when the gene products are processed and signal peptides are removed.

The nucleotide sequences of the presumed promoters and presumptive ribosome binding sites in the *vtx*2ha, *vtx*2hb, *slt*-II and *slt*-IIv operons were identical. These results imply that the *vtx*2h, *slt*-II and *slt*-IIv operons are derived from a common ancestor, that the three operons simply represent a family of operons encoding VT2 and related toxins and that the nucleotide sequences of the A subunit genes are conserved better than those of the B subunit genes. This is in contrast to VT1, whose nucleotide sequences of the genes encoding the A and B subunits are both highly conserved. The products of *vtx*2ha and *vtx*2hb are antigenically indistinguishable and Ito *et al.* (1991) have subsequently demonstrated that the difference in antigenicity between VT2 and VT2va is due to a single amino acid difference in the B subunit of the two toxins.

Recently additional VTEC strains containing two copies of VT2-related genes have been reported and the nucleotide sequences of two different VT2 operons from *E. coli* E32511 determined (Schmitt *et al.*, 1991). One of these operons was determined to be that of *slt*-II while the other, termed *slt*-IIc (table 1.1), in essence contained A subunit sequences from *slt*-II and B subunit sequences from *vtx*2ha (table 1.6). Although VT2vc is closely homologous to VT2va of B2F1, it was found that VT2vc was only slightly less cytotoxic for HeLa cells than Vero cells. Schmitt *et al.* (1991) also proposed that the VT2 that was purified from EHEC strain J2 (Yutsudo *et al.*, 1987) may be impure, as their results suggested the possibility of two *slt*-II genes in this strain.

Table 1.6 **Comparison of the nucleotide and predicted amino acid sequences of the processed A and B subunits of the *slt*-IIc genes with those of the *slt*-II family** (Schmitt *et al.*, 1991)

	Nucleotide sequence homology[a] (%)		Deduced amino acid sequence homology[a] (%)	
Operons[b]	A subunit	B subunit	A subunit	B subunit
slt-IIc	100 (100)[a]	100 (100)	100 (100)	100 (100)
*vtx*2ha	98.5 (98.5)	100 (100)	99.0 (99.1)	100 (100)
*vtx*2hb	98.7 (99.6)	98.6 (98.5)	99.0 (99.1)	100 (98.9)
slt-II	99.7 (99.6)	95.2 (94.8)	100 (100)	97.1 (96.6)
slt-IIv	94.6 (94.4)	79.0 (82.8)	94.2 (94.2)	82.9 (85.4)

[a] Values represent % homology with the indicated sequences from the A & B subunits of *slt*-IIc. Numbers in brackets are homologies comparing unprocessed gene products.

[b] Sequences for *vtx*2ha & *vtx*2hb, *slt*-II, *slt*-IIv and *slt*-IIc were from references; Ito *et al.*, 1990; Jackson *et al.*, 1987b; Weinstein *et al.*, 1988; Schmitt *et al.*, 1991 respectively.

A further VT2 variant has been purified from an *E. coli* O128 strain isolated from a human infant with diarrhoea (Gannon *et al.*, 1990). This strain was described as a VTe variant (VTeva) in view of the fact that it has 98% nucleotide homology with the B

subunit of the VTe gene and only 78.1% homology with the B subunit of the VT2 gene. The A subunit of VTeva was found however, to be equally homologous to those of the VT2 (69.5% homology) and VTe (70.6% homology) genes. VTeva differs from VTe in that it is more heat-stable. No phage could be induced from this strain. VTeva like VTe has a 15 base pair (bp) gap between the A and B subunit genes. Using polymerase chain reaction (PCR) technology this strain showed no specific amplification with VT2, VT2v or VTe primers, indicating that VTeva represents a unique member of the VT family (Tyler *et al.*, 1991). Tyler and colleagues also showed that a variety of class II genotypic combinations are possible. In addition, the presence of VT2v genes is not restricted to strains producing only VT2, as evidenced by one *E. coli* O91:H21 strain which also produced VT1. Although a link has been established between the presence of VT2 genes and disease-causing *E. coli* O157 strains (Ostroff *et al.*, 1989; Tarr *et al.*, 1989), whether multiple copies of *slt*-II operons are responsible for enhanced virulence of an EHEC strain remains to be determined.

Homology of the amino acid sequence of the processed A subunit of VTs with the A chain of Ricin has been noted by several workers (De Grandis *et al.*, 1987; Calderwood *et al.*, 1987; Weinstein *et al.*, 1988a). This finding adds support to the possibility that prokaryotic toxins are evolutionarily related to eukaryotic enzymes, a hypothesis which has been suggested before in relation to diptheria and cholera toxins (Moss & Vaughan, 1978).

1.4.4 Regulation of toxin production

1.4.4.1 High- and low- level production of VTs

VTEC produce variable amounts of toxin and in a study to determine the frequency and levels of VT produced by a wide variety of *E. coli* strains, Marques *et al.* (1986) examined culture supernatants and sonic lysates prepared from these strains for

cytotoxic effects on HeLa cells. Cytotoxic titres expressed as the reciprocal of the cytotoxic dose required to kill 50% of HeLa cells (CD_{50}), per ml of sonic lysate were defined as follows : low (2×10^1 to 6×10^2 CD_{50}), moderate (10^3 to 10^4 CD_{50}), or high (10^5 to 10^8 CD_{50}). It was discovered that although all the *E. coli* strains made at least small amounts of cell-associated toxin, only the *E. coli* strains which produced elevated (moderate or high) levels of VTs were associated with cases of diarrhoea, HC and HUS. This would suggest that cytotoxin may only be a virulence determinant for *E. coli* when it is produced in large amounts. *E. coli* that produce elevated levels of VTs most often belong to a restricted number of serotypes, including O157:H7, O26:H11 and O111:NM. High-level toxin-producing strains are readily identified as VTEC and give clear results in toxin neutralisation tests. It has been found that ShT and VT1 are associated with organisms that produce high levels of cytotoxin while VT2 and VTe are associated with organisms that produce moderate levels of cytotoxin (Strockbine *et al.*, 1986). One possible explanation for the lower levels of cytotoxin produced by the VT2 and VTe strains could be that the strengths of their promoters may be weaker than that of the *stx*/*slt*-I promoter (section 1.4.4.2).

1.4.4.2 Transcriptional efficiencies

In contrast to the VT1/ShT promoter the nucleotide sequence of the VT2 promoter is not closely homologous to a consensus sequence established from a compilation of defined *E. coli* promoters (Sung *et al.*, 1990). Promoters which lack homology with the consensus sequence are generally less efficient at initiating transcription than promoters with a high degree of homology. Sung *et al.* (1990) hypothesised therefore, that the *slt*-I promoter has a more effective transcription start signal than the *slt*-II promoter. In support of this hypothesis was the observation that the cytotoxic activities observed in the cell lysates of VT1-producing EHEC strains are 100- to 1,000-fold higher than for VT2 or VTe producing strains (O'Brien & Holmes, 1987). During comparison of the transcriptional efficiencies of the ShT operon (*stx*) and *slt*-II promoters in fusions to the

chloramphenicol acetyltransferase gene, however, Sung *et al.* (1990) found that the constitutive activity of the *slt*-II/*slt*-IIv promoter was comparable to that of the *stx*/*slt*-I promoter under low iron growth conditions. Therefore, it is likely that the significant differences observed in the production of VT1 and VT2 by toxigenic clinical isolates of *E. coli* are not directly related to differences in the transcriptional efficiences of the *stx*/*slt*-I and *slt*-II/*slt*-IIv promoters.

1.4.4.3 Iron-mediated regulation

Production of ShT has long been known to be increased when cells are grown in medium of low iron concentration. Similarily, iron chelation has been reported to dramatically increase transcription of VT1 and result in an increased concentration of VT1 in the extracellular medium (De Grandis *et al.*, 1987). However, conflicting results for the demonstration of iron-regulation of the *slt*-I operon have been obtained under several experimental conditions. Iron regulation was demonstrated when the *slt*-I genes were present on prophage in lysogenic cells (Weinstein *et al.*, 1988b), or on a high-copy-number *slt*-I::Tn*phoA* fusion plasmid (Calderwood & Mekalanos, 1987) but not when the *slt*-I genes were present on the pBR328 vector (Weinstein *et al.*, 1988b). ShT production in *S. dysenteriae* type 1 strain 60R has been found to also be regulated by temperature, reduced growth temperatures leading to a decrease in ShT production. VT1 production on the other hand is not affected by temperature (Weinstein *et al.*, 1988b).

The regulation of the *slt*1 operon by iron depends on a separate chromosomal locus of the host bacterium, the *fur* gene (Betley *et al.*, 1986; Calderwood & Mekalanos, 1987). The Fur protein is thought to function with iron as a corepressor to negatively regulate toxin production by binding to putative operator sequences and inhibiting transcription of VT1. A recognition site has been located in promoter regions of the VT1 and ShT operons for the *E. coli fur* gene product (Calderwood & Mekalanos, 1987). Other

phage-encoded toxin genes are known to be regulated by iron. For example, expression of the diphtheria toxin operon of the β phage of *Corynebacterium diphtheriae* has been shown to be directly regulated by the iron-dependent binding of a bacterial factor, DtoxR, to the *tox* promoter region (Murphy *et al.*, 1976; Fourel *et al.*, 1989). Iron regulation of the *tox* promoter in *E. coli* had previously been shown to be dependent on the *fur* gene product (Tai & Holmes, 1988).

Iron is essential for the growth of most bacteria and thus specific mechanisms for its aquisition are present in most microbes. In addition to ShT and VT1, the Fur protein represses a variety of iron scavenging systems when sufficient iron is present in the medium. These include genes responsible for the biosynthesis of iron-binding ligands (siderophores) and genes encoding inner and outer membrane proteins involved in the binding and uptake of iron-siderophore complexes. In animal hosts, iron is sequestered by iron-binding proteins and as a result there is little free iron available, in contrast to the comparably high levels of free iron present in the environment. The shift from a high- to low-iron environment may serve through regulatory proteins such as Fur, as a signal to the bacterium that it has entered the mammalian host, triggering the coordinate expression of a number of virulence determinants.

Iron is found not to suppress VT2 or VTe production and neither of their putative promoter sequences show any sign of homology to a binding site for the Fur protein (Sung *et al.*, 1990). This indicates that the regulation of VT2 and VTe synthesis differs from that of ShT and VT1.

1.4.4.4 Kinetics of VT production

In an attempt to maximise VT1 production, Chart *et al.* (1987) examined the effect of iron-restricted/limited growth on VTEC with an aim to increasing yields of VT and enhancing the sensitivity of detection of low-level VT-producing strains of *E. coli*. The

kinetics of VT production indicated that toxin was secreted throughout exponential growth with highest VT titres correlating with highest cell density. In contrast to De Grandis *et al.* (1987) and Weinstein *et al.* (1988b), Chart and colleagues found that iron-restriction led to poor growth and reduced yields of VT1 and that there was no advantage to be gained by growing bacteria under iron restriction/limitation.

1.4.4.5 Mitomycin C induction of VT's

Head *et al.* (1988a) demonstrated an increase in VT2 production following addition of mitomycin C to the culture medium. This induction has been utilised in conjunction with P1 glycoprotein affinity chromatography to purify large amounts of VT1 and VT2 from *E. coli* C600 (933J/W) (Donohue-Rolfe *et al.*, 1989). The mechanism whereby mitomycin C increases VT1 and VT2 production is thought to be via the induction of bacteriophage, resulting in an increase in the copy number of toxin genes (Acheson *et al.*, 1990). In contrast, mitomycin C has no effect on toxin production from *S. dysenteriae* type 1 strain 60R, in which the toxin genes are not phage associated. Another known inducer of phage replication, UV irradiation, also increases both phage and toxin production in irradiated cultures of C600 (933W), (Acheson *et al.*, 1991). Additional proof that mitomyin C induces toxin via its effect on phage replication has been obtained by introducing transforming phage into *E. coli* strains with a *recA*$^-$ background (Acheson *et al.*, 1991). Phage induction is known to depend on the RecA gene product, which also functions in DNA repair responses. DNA damage, whether due to UV energy or alkylating agents such as mitomycin C, activates the RecA protease, which cleaves the LexA gene product. LexA is a repressor of several unlinked genes that encode DNA repair proteins. When LexA is cleaved these so-called damage-inducible (*din*) genes are de-repressed. RecA protease also cleaves phage repressor proteins, thus leading to the transition from the lysogenic to the lytic phase. In a *recA*$^-$ *E. coli* containing the VT-transforming phage it was found that mitomycin C was an ineffective inducer, indicating that the same control mechanism is applicable to VT-

transforming phage. In an attempt to optimise conditions for VTe production from a strain containing the cloned genes for VTe, TB1 (pCG6), MacLeod and Gyles (1989) grew this strain in the presence of mitomycin C and found that the predominantly cell associated toxin in this strain was induced and released into the culture medium. TB1 (pCG6) does not possess a phage toxin gene and the mechanism for toxin induction by mitomycin C in this strain remains uncertain.

1.5 Distinction of VTEC from other pathogenic *E. coli*

E. coli is part of the normal microflora of the intestinal tract of human beings and of most warm blooded animals. Generally the strains that colonise the human bowel are harmless commensals. However, there are pathogenic strains that cause distinct syndromes of diarrhoeal disease and that have been associated with foodborne illness. These foodborne pathogens are grouped into four major categories, summarised in figure 1.3 and described in detail in sections 1.5.1 to 1.5.4. These categories are based on distinct virulence properties, different interactions with the intestinal mucosa, distinct clinical syndromes, differences in epidemiology, and distinct O:H serogroups (Levine & Edelman, 1984; Robins-Browne, 1987; Levine, 1987).

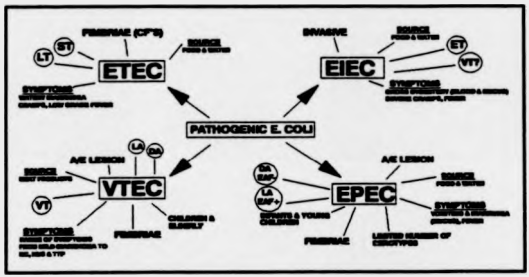
1.5.1 Enterotoxigenic *E. coli* (ETEC)

ETEC are a major cause of bacterial diarrhoea in third world countries and of travellers' diarrhoea. ETEC infection is aquired principally by ingesting contaminated food or water and the clinical features are watery diarrhoea, nausea, abdominal cramps and low grade fever.

After ingestion, ETEC cells that have survived the hostile environment of the stomach overcome the peristaltic defense mechanism of the small intestine by adhering to the mucosa by means of specific fimbriae or colonisation factors. They are non-invasive

and produce one or both of two plasmid encoded cholera-like toxins, LT-I or heat stable toxin (ST). It is the specific action of LT-I or ST on the enterocyte at the subcellular level that leads to fluid secretion, which results in watery diarrhoea in the patient. A further heat-labile toxin (LT-II) has now been described (Guth *et al.*, 1986; Holmes *et al.*, 1986) which has biological activities similar to LT-I but is antigenically distinct. Additionally the structural genes for LT-II are not encoded by plasmids.

Figure 1.3 A summary of the main characteristics of pathogenic *E. coli*



Pathogenic *E. coli* are grouped into four major categories; enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and verotoxin-producing (VTEC), based on a number of distinguishing characteristics. These characteristics are summarised above and discussed in more detail in sections 1.5.1 to 1.6.2. ETEC elaborate one or both of two plasmid encoded toxins, heat labile (LT) and heat stable (ST) enterotoxins, VTEC produce verotoxin (VT) in their culture supernatants and EIEC produce a novel enteroinvasive enterotoxin (EC), in addition to low levels of VT's which are distinct from the VT's of VTEC. VTEC and EPEC both produce characteristic attaching and effacing (A/E) lesions *in vivo* and demonstrate localised (LA) and diffuse (DA) adherence to HEp-2 cells in tissue culture. LA in EPEC has been shown to be encoded by an EPEC adherence plasmid (EAF).

1.5.2 Enteroinvasive *E. coli* (EIEC)

EIEC are an important cause of diarrhoeal disease and dysentery in young children in less developed countries and of food-borne outbreaks of enteric disease in adults in industrialised countries. For most food-related outbreaks, sources of EIEC contamination are associated with an infected foodhandler or contact with water that has probably been contaminated with sewage. Clinical symptoms include fever, severe abdominal cramps, malaise, toxaemia and watery diarrhoea followed by gross dysentery consisting of scanty stools of blood and mucus.

EIEC do not produce LT or ST but, like *Shigellae*, with which they share antigens, they have the ability to invade and multiply within the colonic epithelial cells and cause eventual death of the cell. These strains are able to produce inflammatory keratoconjunctivitis in the eye of a guinea pig (the Sereny test) and this invasive property depends on the presence of a large, approximately 140 megadalton (MDa) enteroinvasive plasmid (pInv) coding for the production of several outer membrane proteins involved in the invasion of mammalian cells (Harris *et al.*, 1982; Hale *et al.*, 1983). These polypeptides are encoded on a 22 MDa section of the large virulence plasmid and this segment is shared and highly conserved in the virulence plasmids of all *Shigellae* and EIEC (Hale & Formal, 1986). Recently it has been found that all major EIEC O serogroups produce low levels of VTs that are immunologically and genetically distinct from VT1 and VT2 of EHEC, (Fasano *et al.*, 1990). This corroborates earlier findings that EIEC strains elaborate cytotoxins that are not neutralised by anti-VT1 or anti-VT2 antibodies (Cleary & Murray, 1988; Marques *et al.*, 1986). The significance of low-level cytotoxin production is uncertain. While epithelial cell invasiveness is clearly the predominant virulence property reponsible for the dysenteric syndrome, presence of these cytotoxins may dictate the severity of the symptoms. The EIEC strains tested by Fasano *et al.* (1990) were also found to produce an enterotoxin of approximately 68-80 KDa, distinct from the EIEC cytotoxin. This novel enteroinvasive enterotoxin (EIET) is

thought to be responsible for the watery diarrhoea which typically precedes dysentery in EIEC infection.

1.5.3 Enteropathogenic *E. coli* (EPEC)

EPEC strains belong to a limited number of serotypes and were the first *E. coli* strains to be implicated epidemiologically in diarrhoeal disease. An isolate is classified as an EPEC strain if it is isolated from a case of diarrhoea and belongs to one of the following O serogroups: O55, 086, O111, O114, O119, O125, O126, O127, O128, O142, O158. Clinically EPEC infection occurs most frequently in infants and young children and is characterised by fever, vomiting and diarrhoea with prominant amounts of mucus but without gross blood.

The mechanism by which EPEC cause disease remains to be defined. EPEC are not enteroinvasive and do not produce the classical LT or ST, although some strains have been shown to produce other toxins (Scotland *et al.*, 1980; O'Brien *et al.*, 1982; Katouli *et al.*, 1989). In a later study by Cleary *et al.* (1985) it was found that EPEC strains, particularly those of serogroup O26, commonly produce VT. Although it was found that the amount of cytotoxin produced by EPEC is significantly higher than that produced by strains from healthy individuals, the biological significance of this remains unclear, since there is considerable overlap in the range of concentrations found. Adherence of many enteric pathogens to intestinal mucosal surfaces is a critical primary step in the pathogenesis of diarrhoeal disease. The importance of adherence factors in EPEC and VTEC are discussed in detail in section 1.5.5.

1.5.4 Verotoxin-producing and enterohaemorrhagic *E. coli* (VTEC and EHEC)

VTEC refers to all *E. coli* strains that produce VT in their culture supernatants and can therefore be distinguished from other classes of *E. coli* that do not do so. Although the

vast majority of *E. coli* strains do not make VT, it is common for both healthy infants and adults to have VTEC as part of their normal faecal flora (Cleary *et al.*, 1985). VTEC are not enteroinvasive (by the Sereny test) and do not produce the classic LT and ST. The term EHEC refers to strains that have the same clinical, epidemiological and pathogenic features associated with the prototype EHEC organism, *E. coli* O157:H7 described by Riley *et al.* in 1983 (Levine, 1987). Only two EHEC serotypes (O157:H7 and O26:H11) have been classified by Levine, 1987. EHEC strain O26:H11 (H30) was previously categorised as an EPEC. Thus EHEC constitute a defined theoretical subgroup of VTEC.

1.6 Additional virulence determinants

Virulence factors are bacterial products that are required by the organism to cause disease. Deletion of any of these factors results in a reduction in, or loss of virulence.

In addition to toxin production, adherence of many enteric pathogens to intestinal mucosal surfaces is a critical primary step that results in bacterial colonisation of the intestine necessary for both delivery of elaborated toxins to enterocytes and tissue invasion. Enteroadherence of bacteria promotes increased colonisation of organisms since they are less susceptible to distal clearing by the peristaltic activity of the gut. Adherence can be mediated by various cell surface antigens including fimbriae (pili), flagella, outer membrane proteins, capsular polysaccharide and lipopolysaccharides (LPS).

Gram-negative bacteria, including ETEC and EPEC adhere by means of fimbriae. Adherence of fimbriae to specific receptors on the surface of the cell is the basis for the agglutination of erythrocytes or red blood cells (RBC) by fimbriated bacteria. The agglutination of guinea pig RBC by some strains of *E. coli* can be blocked with D-mannose or its analogues. These mannose-sensitive adhesins (type 1 fimbriae) are

found on 50-70% of all *E. coli* isolates and are particularly prevalent among normal flora isolates of *E. coli*. By contrast most strains of *E. coli* that are associated with intestinal or extraintestinal disease in humans agglutinate guinea pig RBC even in the presence of mannose (Salit *et al.*, 1983). Whereas most other fimbriae produced by *E. coli* gastrointestinal pathogens are encoded on plasmids, type 1 fimbriae are chromosomally encoded. The presence of fimbriae by itself is not a marker for virulence since nonvirulent commensals tend to be much more fimbriated than pathogens when grown in broth, but the numbers of fimbriae on bacteria varies with the growth conditions employed (Salit *et al.*, 1983).

1.6.1 Adherence of EPEC

1.6.1.1 HEp-2 cell adherence

In 1979, Cravioto *et al.*, showed that the majority of EPEC outbreak strains could adhere to HEp-2 human epithelial cells in tissue culture in the presence of D-mannose. Attachment of these outbreak strains to HEp-2 cells was later found to be encoded on a large 55-60 MDa plasmid (Baldini *et al.*, 1983). The name EPEC adherence factor (EAF) was given to this property and through volunteer studies the EAF adhesion system was shown to be important in the ability of EPEC to cause diarrhoea. EPEC adhere to HEp-2 cells in two distinct patterns, termed localised adherence (LA) and diffuse adherence (DA), (Scaletsky *et al.*, 1984; Nataro *et al.*, 1985a). EPEC showing LA, adhere in discrete microcolonies to localised areas of the HEp-2 cell surface, whereas those showing DA adhere to the entire cell surface. LA and DA are encoded for by different plasmids and use of a 1 kilobase (Kb) EAF DNA probe derived from a 55 MDa EAF plasmid, pMAR2 from strain E2348, showed that the LA phenotype was EAF plasmid encoded (Nataro *et al.*, 1985a). Nataro *et al.* (1985b) subdivided the EPEC category into two classes on the basis of hybridisation with the 1 Kb EAF probe. Class I EPEC (EAF^+) exhibit LA to HEp-2 cells, whereas class II EPEC (EAF^-)

exhibit either DA or no adherence at all to HEp-2 cells. Both class I and class II EPEC are associated with diarrhoeal disease. An ELISA using antiserum to EAF plasmid-associated factors has subsequently been developed for the detection of classic EPEC displaying LA (Albert *et al.*, 1991). In diarrhoeagenic *E. coli* strains exhibiting the DA phenotype at least two different adhesins have so far been identified. Bilge *et al.* (1989) have characterised a region of chromosomal DNA coding for a fimbrial adhesin. More recently the cloning and expression of a 6 Kb DNA fragment from a 100 Kb (2 MDa) plasmid harboured by EPEC clinical isolate 2787 which confers the DA phenotype to recipient *E. coli* K12 strains, as well as the identification of a 100 KDa protein (AIDA-I-adhesin involved in diffuse adherence) as the DA-mediating adhesin has been described (Benz & Schmidt, 1989, 1992). Serologically related proteins of similar size were detected by Western blotting in other DA^+ diarrhoea-associated EPEC strains (Benz & Schmidt, 1992).

HEp-2 cell adherence was originally suggested to be a good model of *in vivo* EPEC adherence (Baldini *et al.*, 1983). However, in the original description of HEp-2 cell adherence by Cravioto *et al.* (1979), 29% of 17 non-EPEC, non-ETEC strains that were possible causes of five diarrhoeal outbreaks were adherent to HEp-2 cells. Mathewson *et al.* (1985) reported that nontoxigenic, HEp-2 cell adherent strains of *E. coli* displaying LA or DA and which did not belong to the recognised EPEC serogroups were an important cause of travellers' diarrhoea. They proposed, therefore that HEp-2 cell adherence may be a virulence factor in *E. coli* strains independent of serogroup, and for this reason Mathewson proposed that HEp-2 cell-adherent *E. coli* strains be referred to as enteroadherent *E. coli* (EAEC), in order to avoid reference to and confusion with strains belonging to EPEC serogroups. More recently evidence suggests that EAEC are identifiable by a particular pattern of adherence to Hep-2 cells that is clearly distinguishable from both LA and DA (Levine, 1987).

1.6.1.2 Attaching and effacing activity

In vivo studies have shown that EPEC strains adhere avidly to the mucosa of the small intestine with local destruction of microvilli and pedestal formation (Moon *et al.*, 1983). The characteristic attaching and effacing (A/E) lesion produced is thought to be responsible for the diarrhoea associated with EPEC (Robins-Browne, 1987) and strains producing this type of lesion have been referred to as attaching and effacing *E. coli* (AEEC).

Levine *et al.* (1985) showed that a 94 KDa outer membrane protein was detected in wild type EPEC strains but not in EAF plasmid-cured derivatives of these strains, suggesting that the 94 KDa membrane protein of EPEC may be encoded for by the EAF plasmid. However, an isogenic EPEC strain cured of the pMAR2 plasmid continued to demonstrate A/E adherence to HEp-2 cells (Knutton *et al.*, 1987a) and enterocytes in organ culture (Knutton *et al.*, 1987b). Others have also indicated that adherence of EPEC to HEp-2 cells does not depend on the expression of fimbrial adhesins (Scotland *et al.*, 1983b). Knutton *et al.* (1987a, b) provided evidence that the LA to HEp-2 cells and the A/E adherence are two genetically distinct phenomena. They proposed a two stage model for EPEC adherence with a fimbrial mediated initiation step (plasmid encoded and responsible for HEp-2 LA) and a subsequent phase of A/E adherence (encoded for by genes on the chromosome). It was evident that the second stage can occur in the absence of the first stage, but the presence of plasmid-encoded adhesins appears to greatly enhance the ability of EPEC to colonise the mucosa (Tzipori *et al.*, 1989). In addition to enabling initial colonisation, the EAF adherence plasmid may provide tissue specificity for human epithelial cells (Jerse *et al.*, 1991). Studies using Tn*phoA* mutagenesis indicated that the 94 KDa outer membrane protein was encoded by a chromosomal locus (*eae* - *E. coli* A/E). Furthermore the *eae* gene has been shown to be necessary for A/E activity on human tissue culture cells (Jerse *et al.*, 1990) and to share significant homology to invasin, a known virulence factor of *Yersinia*

pseudotuberculosis (Jerse & Kaper, 1991). Expression of the *eae* gene was found to be positively regulated by the EAF plasmid (Jerse & Kaper, 1991). This may explain the earlier observations that *in vivo* (Knutton *et al.*, 1987b) and *in vitro* (Tzipori *et al.*, 1989), increased numbers of A/E lesions are produced by EPEC strains that possess the EAF plasmid.

1.6.2 Adherence in VTEC

Before 1987 the surface antigens which mediate adherence of *E. coli* O157:H7 to epithelial cells had not been defined, although adherence of *E. coli* O157:H7 to the colons of orally infected gnotobiotic piglets (Tzipori *et al.*, 1986), infant rabbits (Pai *et al.*, 1986) and chickens (Beery *et al.*, 1985) had been described. *E. coli* O157:H7 had also been shown to adhere to the surface epithelial cells in the intestines of postweaning rabbits (Sherman *et al.*, 1988). VTEC of multiple serotypes have been shown to possess the ability to cause A/E lesions (Sherman *et al.*, 1988), however, bacterial A/E activity caused by these VTEC strains has not yet been shown to be an important virulence factor in the large bowel in humans, since it would require biopsies to be taken from the ileocaecal region during acute infection.

Sherman *et al.* (1987) found that VTEC O157:H7 strains exhibit LA and/or DA to HEp-2 cells and to Henle 407 gut-derived epithelial cells in tissue culture. In later studies they provided evidence that specific constituents of O157:H7 outer membranes rather than O157 LPS or H7 flagellin, mediated adherence of the organisms to HEp-2 cells in tissue culture (Sherman & Soni 1988; Sherman *et al.*, 1991). In this study a 94 KDa outer membrane protein was implicated as a bacterial adhesin involved in A/E activity. The similarity in size and cellular location between this protein and the 94 KDa *eae* protein of EPEC suggests that the protein described by Sherman *et al.* (1991) may be the *eae* protein of that strain (Jerse & Kaper, 1991). Jerse *et al.* (1990, 1991) showed that all EPEC and EHEC displaying A/E activity possess chromosomally

encoded sequences homologous to a 1 Kb *eae* probe. Although these proteins may serve similar functions in the production of A/E lesions, they differ antigenically.

In contrast to Sherman *et al.* (1987), adherence of VTEC O157:H7 strains to Henle 407 cells but not to HEp-2 cells has been reported (Karch *et al.*, 1987). Evidence was provided that adherence to the Henle 407 cell line was determined by non-haemagglutinating, mannose-resistant fimbriae encoded for by genes on a 60 MDa plasmid. In addition, small numbers (typically two to four) of O157:H7 bacteria were shown to attach to Henle 407 cells in a central location, whereas plasmid-cured derivatives failed to adhere. This data conflicts with evidence that a plasmidless derivative of an *E. coli* O157:H7 strain adhered to Henle 407 cells three times better than the parent strain (Junkins & Doyle, 1989) and a plasmidless derivative of serotype O157:H7 caused typical A/E lesions in the piglet intestine (Tzipori *et al.*, 1987, 1989). In addition, a plasmid cured derivative of *E. coli* O157:H7 strain 933, which lacks a 60 MDa plasmid but contains other plasmids, colonised streptomycin treated mice in high numbers when fed to mice (Wadolkowoski *et al.*, 1990a). However *E. coli* O157:H7 strain 933 colonised better than its plasmid-cured derivative when the organisms were fed simultaneously to streptomycin-treated mice. These results suggest that the 60 MDa plasmid may encode a factor or factors required for *E. coli* O157:H7 strains to colonise in the presence of other bacteria. This factor may be the plasmid-encoded fimbriae which mediate attachment to Henle 407 intestinal cells *in vitro*. The inconsistency of these observations would seem to suggest that the genetic basis for adherence to epithelial cells in tissue culture is very variable (Hall *et al.*, 1990) and may be different from that reported for strains of serotype O157:H7 published by Karch *et al.* (1987). Use of a 3.4 Kb segment of this plasmid as a DNA probe, termed CVD419, in a survey showed that VTEC strains commonly harbour this plasmid, (hybridisation with 99% VT^+ O157:H7, 77% VT^+ O26:H11 & 77% other VT^+ *E. coli*), whereas commensal *E. coli* and strains belonging to other classes of pathogenic *E. coli* gave a negative reaction (Levine *et al.*, 1987), making it a useful screening test for epidemiologic and diagnostic

purposes. Levine *et al.* (1987) also showed that VTEC O157:H7 strains, unlike class I EPEC strains, are EAF probe negative.

1.6.3 Flagella

Flagella are filamentous protein appendages that protrude from the cell surface of many bacteria and confer motile properties to the organisms. As a result of motility, flagella may permit increased bacterial penetration of the surface mucous gel and thus promote increased colonisation by enteric pathogens at the mucosal surface. In addition to virulence associated with motility properties of bacteria, certain flagella may also function as bacterial attachment factors or adhesins that promote binding of pathogenic organisms to receptors present on the surface of enterocytes and colonocytes. Although Sherman & Soni (1988) showed H7 flagella not to be important in adherence to human epithelial cells, H7 antigens nevertheless appear to be an important factor in the pathogenesis of human disease because nonmotile, aflagellar *E. coli* strains of the O157 serogroup are much less frequently isolated in stool specimens from symptomatic patients than are *E. coli* strains of the O157:H7 serotype.

From the early days of the 1940's when *E. coli* were first convincingly associated with human diarrhoea, much has been discovered about the several categories of diarrhoeagenic *E. coli*, including information on their clinical features, epidemiology, O:H serotypes, and most particularly their pathogenesis. New virulence factors are constantly being discovered which can be found to span several diarrhoeagenic *E. coli* serogroups. Johnson & Lior (1988) described a new heat-labile *E. coli* toxin, cytolethal to Vero, HeLa, HEp-2 and CHO cells and negative in Y1 cells in the culture filtrates of 43 *E. coli* strains associated with diarrhoeal disease. The toxin, termed a cytolethal distending toxin (CLDT) was distinct from the classic LT and ST enterotoxins, VT's and hemolysins. However VT and LT were found in combination with CLDT in a few *E. coli* strains. As knowledge has grown it has become increasingly evident (Guth *et al.*,

1989), that the ability of *E. coli* to cause intestinal infection is associated with bioserotypes rather than with O serogroups. It has been observed that the same *E. coli* serogroup may harbour enterotoxigenic and enteroadherent serotypes (Guth *et al.*, 1985), enterotoxigenic and invasive serotypes (Gross *et al.*, 1983), cytotoxigenic and enteroadherent serotypes (Bopp *et al.*, 1987) and cytotoxigenic and enterotoxigenic serotypes (Marques *et al.*, 1986; Katouli *et al.*, 1989). An O138 strain has been shown to harbour the genes for cytotonic ST in addition to VTe (Meyer & Karch, 1989). Coexpression of two potent toxins may enhance the virulence of these strains in the host.

1.7 Clinical and public health significance of VTEC infections

Evidence that VTs are involved in the pathogenesis of diarrhoeal disease is predominantly circumstantial. There is, however, strong epidemiological evidence for this and in a substantial review of VTEC, Karmali (1989) documents numerous published studies of outbreaks and sporadic cases of VTEC infection, due in most cases to serotype O157:H7 that indicate the spectrum of illness includes asymptomatic infection, mild uncomplicated diarrhoea, HC, HUS and thrombotic thrombocytopaenic purpura (TTP), a syndrome that is closely linked to HUS. The severe features of HC, HUS and TTP characterise VTEC as more important pathogens than the other types of EPEC.

1.7.1 Haemorrhagic colitis (HC)

HC was defined as a clinically distinct entity approximately 20 years ago. It is distinguished from inflammatory colitis by a natural progression from watery to bloody diarrhoea over the course of a few days and a relative lack of the usual signs of inflammation such as fever and large numbers of pus cells in the stool. The cause of the syndrome was unknown until 1982 when two separate outbreaks of HC in Michigan and

Oregon were investigated by the Centers for Disease Control and led to the discovery of a particular serotype of *E. coli*, O157:H7, as an etiologic agent (Riley *et al.*, 1983). Historically, O157:H7 has been a serotype that was rarely isolated from humans and animals. The Centers for Disease Control (Atlanta) detected only a single strain of O157:H7 in more than 3000 isolates serotyped in the period from 1973 to 1982 (Riley *et al.*, 1983); the *E. coli* Reference Center at Pennsylvania State University reported no serotype of O157:H7 in more than 20,000 serotyped cultures isolated from animals (Riley *et al.*, 1983); and in Great Britain, only one O157:H7 strain was identified in more than 15,000 strains serotyped before 1983 (Day *et al.*, 1983). The study by Riley *et al.* (1983) attracted widespread attention and set the stage for several additional studies to understand better the magnitude of the problem. It soon began to emerge that *E. coli* O157:H7 was by no means a rare serotype as initially thought but rather was a fairly common isolate from patients with HC and unspecified bloody diarrhoea. A survey in England and Wales showed that VTEC were responsible for 39% of sporadic cases of HC (Smith *et al.*, 1987) and of these, 25% belonged to serotype O157:H7. Studies have shown however, that VTEC associated with HC include atypical O157 strains and other serotypes.

Although the precise role of VTs in HC is not fully understood there is substantial evidence that the toxins are important virulence factors in the development of bloody, oedematous vascular lesions of the colon (Fontaine *et al.*, 1988). The toxins may also participate in the direct killing of colonic epithelial cells and may provoke fluid secretion and diarrhoea in the host (O'Brien & Holmes, 1987).

1.7.2 Haemolytic uraemic syndrome (HUS)

First described in 1955 as a distinct clinical entity, HUS is characterised by acute renal failure, thrombocytopaenia and haemolytic anaemia. The disease may present in several forms but in most cases a prodromal illness of bloody diarrhoea precedes the

onset of the disease. HUS occurs in all age groups but represents the commonest cause of acute renal failure in children. In recent years increasing numbers of cases have been observed in localised epidemics during the summer months. HUS is rare in adults but it is not exclusively a disease of childhood (Crosse & Naylor, 1990). It has been reported in a variety of clinical and epidemiological settings and several different agents, including drugs, chemicals, toxins and microbes have been postulated as potential causes.

In a preliminary communication, Karmali *et al.* (1983) first reported that VTEC as well as free VT were present in the stools of some patients with HUS. Further studies indicated that VTEC have a close and probably causal association with HUS (Karmali *et al.*, 1985a), a finding which has subsequently been corroborated by many investigators (Karmali, 1989). In Britain, *E. coli* O157:H7 has commonly been associated with outbreaks and sporadic cases of HUS (Taylor *et al.*, 1986; Scotland *et al.*, 1988; Milford & Taylor, 1990; Milford *et al.*, 1990). In the states of Minnesota and Washington VT2 producing *E. coli* O157:H7 are the most common isolates from HUS patients (Ostroff *et al.*, 1989; Martin *et al.*, 1990). In contrast, in Buenos Aries which probably has the highest reported incidence of HUS in the world, approximately equal numbers of non-O157:H7 VTEC producing VT1, VT2 and both toxins are the principal causative organisms (Lopez *et al.*, 1989). This last study underscores the importance of looking for VTEC strains in patients with HUS using a technique which is not specific for serotype O157:H7.

An interesting situation which adds to the epidemiological base suggesting a possible role of VT's in HUS is that of cancer-associated HUS (C-HUS). This entity has been linked to the use of the drug mitomycin C as a chemotherapeutic agent (Lesesne *et al.*, 1989). Mitomycin is known to induce bacteriophages in bacteria and has been shown to dramatically increase the level of VT1 and VT2 produced *in vitro* (section 1.4.4.5). It is possible therefore, that therapeutic levels of mitomycin C during cancer treatment may

also exert a similar inductive effect on VTEC that may be present in the gut flora (Acheson & Donohue-Rolfe, 1989). If so, then *in situ* induction of VT production may initiate or contribute to development of C-HUS, whereas in the absence of the inducing agent these organisms do not produce sufficient toxin to be pathogenic. Individual susceptibility may also be important and the value of antimicrobial treatment during illness remains conflicting. Studies by Ryan *et al.* (1986) suggested that antimicrobial therapy was ineffective, whereas some authors have found that antimicrobial treatment during the prodromal illness was associated with a mild clinical course and a good outcome (Martin *et al.*, 1990). Others have reported a detrimental effect of antimicrobial use before hospitalisation for cases of HUS associated with infection with *E. coli* O157:H7 (Carter *et al.*, 1987). Also laboratory evidence suggests that some antimicrobial agents (such as trimethoprim-sulfamethoxazole) enhance the release of VT from *E. coli* (Karch *et al.*, 1985). It has also been suggested that use of antimotility agents in the treatment of VTEC infections may result in increased mortality and morbidity (Ryan *et al.*, 1986). Decreased intestinal motility may allow multiplication of the organism and increased production of VT.

The mechanism by which VT may be involved in the pathogenesis of HUS is at present unknown, although accumulating evidence suggests that the toxins may have a direct cytotoxic effect on endothelial cells (Obrig *et al.*, 1988; Milford & Taylor, 1990). One study reports that VT decreases prostacyclin synthesis by endothelial cells (Karch *et al.*, 1988). Prostacyclin is important in regulating intravascular hemostasis and platelet aggregation. Furthermore, VT2 was seen to reduce prostacyclin synthesis more than VT1 and may reflect the relative potencies of these toxins in cell toxicity. Histopathologic studies of the kidneys of HUS patients have shown profound alterations in the glomeruli and it has been hypothesised that VT's produced by the VTEC strains are disseminated from the gut by the blood system and targeted to the kidneys where they generate damage to the glomerular endothelial cells (Wadolkowski *et al.*, 1990b). Human vascular endothelial cells have been shown to be relatively resistant to ShT and

VT's (Tesh *et al.*, 1991). This may be due to reduced toxin binding, as low levels of Gb_3, the VT1 and VT2 toxin-specific receptor (section 1.3.3.1), were found in human vascular endothelial cell membranes whereas Gb_3 is a predominant neutral glycolipid in human renal tissue (Boyd & Lingwood, 1989). Boyd & Lingwood (1989) have also shown that except for the pig, renal tissue from various animals lack Gb_3. This is consistent with the lack of renal lesions in animals given VT or VTEC.

1.7.3 Thrombotic thrombocytopaenic purpura (TTP)

First described by in 1924, TTP is an uncommon disease of young adults and closely resembles HUS in its clinicopathological features but differs in that neurological signs and fever are more prominant in TTP. A pentad of clinical features are associated with TTP. These include, fever, thrombocytopaenic purpura, microangiopathic haemolytic anaemia, neurological manifestations which are often remittent and renal dysfunction. Most cases of TTP present without an antecedent illness, whereas a prodromal diarrhoeal illness is an essential feature of classical HUS. Recently two cases of TTP have been associated with *E. coli* O157:H7 infection where, unlike the usual form of TTP, the patients had an antecedent bloody diarrhoeal illness and the disease therefore resembled classical HUS (Karmali, 1989).

1.7.4 Outbreaks of VTEC infection

VTEC strains of different serotypes are increasingly isolated from cases of human and animal diseases. Most of them belong to the serotype O157:H7 and surveys have revealed that *E. coli* O157:H7 is responsible for up to 40% of acute infectious diarrhoeas in some areas (Riley, 1987). Following the initial outbreaks of *E. coli* O157:H7 associated HC in Oregon and Michigan, numerous additional outbreaks have been reported and these have provided new insights into the natural history and epidemiology of VTEC infection.

It has become evident from outbreak studies that VTEC infection results in a highly variable rate of complications with *E. coli* O157:H7-related HUS and TTP more common at the extremes of age. The reasons for this are unclear and may be a function of several host and parasite factors including age, underlying condition (e.g gastrectomy), previous antibiotic treatment, presence or absence of specific antitoxic immunity, the inoculum size of the organism and strain characteristics may also play a role (see sections 1.4.4.1 & 1.7.2), (Carter *et al.*, 1987). Other potential host factors to be investigated include the presence or absence of specific receptors on target cells and blood group status.

1.7.4.1 Influence of blood group status

The cellular receptor for both VT and ShT has been shown to require a terminal disaccharide, galactose-α(1-4)-galactose (section 1.3.3.1), which in man is linked to ceramide and paragloboside as the blood group antigens Pk and P1 respectively (Lindberg *et al.*, 1987). Approximately 75-80% of the normal caucasian population express blood group P1 on RBC but with varying degrees of antigenic strength and it has been found that there is a tendency for HUS patients, and especially those with more severe disease to be negative or only weakly positive for P1 antigen expression. Additionally expression of various blood group antigens is age related and P1 positive individuals have reduced expression both in their pre-school years and in senescence. It has been hypothesised therefore that in patients exposed to VT, adsorption of free toxin onto RBC may reduce the burden of VT to other target cells. Once internalised within the RBC the toxin will be relatively innocuous as there is no ribosomal activity for the active A subunit to interrupt. The early transfusion of P1 positive RBC cells (or a specifically designed ligand) to bind and thus neutralise circulating toxin may prove to be a rational approach to treatment (Peter Rose, Warwick Hospital, personal communication).

1.7.4.2 Influence of toxin type

Recent studies from Britain and the U.S.A, in which the association of HUS and VTEC were examined, have shown that the majority of *E. coli* isolates produced VT2 either alone or together with VT1 (Scotland *et al.*, 1988). In Washington state, epidemiological studies indicate that *E. coli* O157:H7 strains with only VT2 genes are more likely to cause diarrhoeal disease that later progresses to HUS and TTP than organisms containing genes for VT1 or both VT1 and VT2 (Tarr *et al.*, 1989; Ostroff *et al.*, 1989). This finding was independent of age and antimicrobial use and correlates well with data indicating a critical role for VT2 but not VT1 in renal damage associated with *E. coli* O157:H7 infection of streptomycin-treated mice (Wadolkowski *et al.*, 1990a). Perhaps isolates with only VT2 genes produce greater quantities of VT2 *in vivo* than do isolates with both toxin genes. Another possibility is that the VT2 genotype acts as a marker for other as yet unrecognised virulence characteristics.

It has become clear that the clinical presentation of VTEC infection spans a spectrum, ranging from asymptomatic infection, mild uncomplicated diarrhoea, HC, HUS and TTP to death. In Thailand, VTEC have been found in small numbers in a similar proportion of children with bloody diarrhoea in whom other pathogens were not identified and in controls without diarrhoea (Brown *et al.*, 1989a). It is unclear why VTEC are found in as many well children as ill children but perhaps other cryptic virulence factors in addition to the toxin and adherence factors may be necessary to cause disease. Future studies may identify other factors necessary for VTEC to produce diarrhoea.

1.7.5 Epidemiology of VTEC infection

Most outbreaks of VTEC infection have been associated with serotype O157:H7. Results of multilocus enzyme electrophoresis studies indicate that O157:H7 isolates

from North America are interelated, forming a clonal group that has evolved relatively recently (Whittam *et al.*, 1988). A second major finding by Whittam and colleagues was that the O157:H7 clonal genotypes are only distantly related to other VTEC strains. One implication of the clonal relationship of O157:H7 isolates is that there exists an effective mechanism for the widespread, geographic dispersal of these organisms.

Symptomatic and symptom-free human carriers are presumed to be the principal reservoir of EPEC, EIEC and ETEC strains that cause human illness. These bacteria are present in the intestinal tract of carriers and are excreted in their faeces. Infected foodhandlers with poor personal hygiene or water contaminated by human sewage are sources of food contamination. VTEC however, are widely distributed in the intestines of animals and most outbreaks of *E. coli* O157:H7 have been linked epidemiologically to the consumption of undercooked ground beef, and to a lesser extent to drinking of unpasteurised milk. Studies by Wells *et al.* (1991) suggest that dairy cattle, a source of both raw milk and beef products are a major reservoir of *E. coli* O157:H7 and other VTEC. This data corroborates earlier studies by Montenegro *et al.* (1990) that apparently healthy cattle carry VTEC. In Sri Lanka however, VTEC were recovered significantly more often from cattle and water buffalo calves with diarrhoea than from healthy control animals, suggesting that VTEC may cause illness in cattle as well as in humans (Mohammad *et al.*, 1985, 1986).

Studies by Beery *et al.* (1985) revealed that *E. coli* O157:H7 can readily colonise the caecae of chickens and can be excreted in the faeces for several months. This suggests that chickens may serve as hosts and possibly as reservoirs for *E. coli* O157:H7. In surveys of retail raw meats and poultry (Doyle & Schoeni, 1987), 3.7% of ground beef, 1.5% of pork, 1.5% of poultry, and 2.0% of lamb samples were found to be contaminated with O157:H7, indicating that the bacterium is associated with foods particularly of animal origin and not specifically with beef. Additionally, in a report of the first community outbreak of HC in the UK due to *E. coli* O157:H7, vegetables were

thought to have been the source of infection (Morgan *et al.*, 1988). Raw foods of animal origin may be contaminated with the organism via faecal contact during slaughter or milking procedures and most human infections probably occur as a result of consumption of these foods when undercooked. Ground meats are thought to be more at risk if incompletely cooked and outbreak strains have been recovered from implicated beef products in two outbreaks (Wells *et al.*, 1983; Hockin *et al.*, 1987). The organism is believed to be a surface contaminant and the grinding of the meat would introduce the pathogen to the inside of the patty where undercooking is more likely.

Information currently available indicates that human VTEC infections occur most commonly in the summer among children under five years of age and in the geriatric population (Pai *et al.*, 1988). Because of the disease's sharp summer peak in incidence and the association of the organism with hamburger meat, a common assumption is to link the disease with common summer food preparation practices. Thus the lay press has referred to it as "hamburger syndrome" or "barbecue syndrome".

In October 1988, an outbreak of *E. coli* O157:H7 HC occurred among students attending a Minnesota school (Belongia *et al.*, 1991). This outbreak was unusual because it was the first documented foodborne outbreak associated with consumption of heat-processed meat patties, a product that should be pathogen-free. Large-scale production and distribution for precooked meat products creates a potential for widespread illness and in reponse to this outbreak, specific requirements have been proposed for the manufacture of heat-processed uncured meat patties. In a study of the risk factors for *E. coli* O157:H7 infection in an urban community (Bryant *et al.*, 1989), proper food cooking (and handling) of all animal products, rather than the avoidance of these particular food products was found to be the primary prevention strategy.

While VTEC infection in most cases is probably foodborne there is increasing evidence that VTEC infection can also be acquired through person-to-person transmission (Carter *et al.*, 1987; Karmali *et al.*, 1988).

1.8 Laboratory diagnosis of VTEC infection

The major obstacle in large-scale epidemiological investigations of the incidence of VTEC in diarrhoeal stools, is the lack of a rapid, simple and specific test to detect VTEC and their toxins. The purpose of the clinical microbiology laboratory is to rapidly and accurately provide the clinician with information concerning the presence or absence of a microbial agent that may be involved in an infectious disease process. Traditionally, this involves the isolation of a suspected pathogen from a clinical specimen followed by the identification of the organism by serological or biochemical methods. Depending on the organism, the entire identification process can take anywhere from several days to weeks.

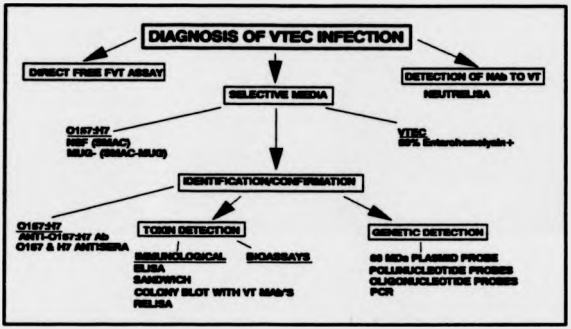
Diagnosis of VTEC infection requires either the isolation of VTEC from stools, the demonstration of neutralisable free faecal VT (FVT) activity in stool filtrates or the detection of rising titres of neutralising antibodies (NAb) to VT in persons suffering from these illnesses. The available detection methods are discussed in detail in the following sections 1.8.1 to 1.8.8 and are summarised in figure 1.4.

1.8.1 Isolation of VTEC from stools

Considerable difficulty has been experienced in isolating VTEC from stools of patients with HUS and HC because the organisms have been present in low numbers. The low or undetectable numbers of VTEC in the stools of some of these patients can partly be explained by the fact that the first stool for investigation is usually received after HUS has been diagnosed and this typically occurs about 1 to 2 weeks after the start of an

acute diarrhoeal disease (Karmali *et al.*, 1985a). Also, findings suggest that faecal VTEC decline in numbers rapidly after the onset of symptoms (Karmali *et al.*, 1985a, 1985b; Wells *et al.*, 1983) and that toxin can be detected in the stool long after the organism has disappeared (Pai *et al.*, 1984).

Figure 1.4 **A summary of the main tests available to diagnose VTEC infections**



Diagnosis of VTEC infection requires either the isolation of VTEC from stools using selective media, the demonstration of neutralisable free faecal VT (FVT) activity in stool filtrates or the detection of rising titres of neutralising antibodies (NAb) to VT in persons suffering from these illnesses. Apart from a close association with enterohemolysin production, the majority of VTEC lack a common phenotypic marker to enable direct detection on selective media. O157:H7 strains however, exhibit characteristics atypical of the *E. coli* group, non-sorbitol fermentation (NSF) and the inability to cleave the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) to a fluorescent end product, methylumbelliferone. Consequently, MacConkey agar which contains D-sorbitol in place of lactose (SMAC) and SMAC-MUG medium have been used to aid in the detection of *E. coli* O157:H7. Confirmation of presumptive VTEC is made using biologiacl or immunological assay methods to screen for the presence of VT. In addition genetic assay methods have been developed to demonstrate the presence of the genes coding for VT's. The various detection methods outlined are discussed in more detail in sections 1.8.1 to 1.8.8.

Lack of a common phenotypic marker among the many different serogroups of *E. coli* said to produce VT's has hampered direct detection of these pathogens in clinical specimens and foods. Beutin *et al.* (1989) has since found that there is a close association, 89%, with VT production and enterohemolysin production in strains of *E. coli*, making enterohemolysin suitable as an epidemiological marker for the rapid detection of potential VT^+ *E. coli* strains. Enterohemolysin is unrelated morphologically, serologically and genetically to α-hemolysin which is found frequently among human faecal *E. coli* strains and predominates in strains causing extraintestinal infections in humans.

Detection of VTEC on routine enteric media is time consuming and expensive. Biological or immunological assay methods have been employed to screen VTEC strains in mixed stool cultures for the presence of VT either in culture supernatants or directly in stool filtrates. In addition, genetic assay methods have been developed to demonstrate the presence of the genes coding for VT's in VTEC. However, the perfect solution - a simple, accurate, reproducible, cheap and sensitive analytical procedure is yet to be devised.

1.8.2 Screening media for the detection of *E. coli* O157:H7

E. coli O157:H7 has been found to exhibit characteristics atypical of the *E. coli* group of organisms. These traits have been used as strain markers and have facilitated detection of O157:H7 in mixed flora from faeces on the appropriative selective media.

In 1982, in the two outbreaks in the U.S.A, it was noted that *E. coli* O157:H7 failed to ferment sorbitol for 48 hours or more (Wells *et al.*, 1983). March & Ratnam (1986) subsequently evaluated the usefulness of MacConkey agar which contained D-sorbitol in place of lactose (SMAC) as a strain marker to aid in the detection of *E. coli* O157:H7 in stool cultures. Non-sorbitol fermenters (NSF) yield colourless colonies on SMAC

(similar in appearance to non-lactose-fermenting colonies on MacConkey agar) and contrast well with the bright pink colonies of sorbitol-fermenting/positive (sor^+) organisms of the faecal flora. They found that only 15% of faecal enterobacteria are NSFs and as 93-95% of all *E. coli* are sor^+, this led to the widespread use of SMAC for screening stool specimens. There has however, been one published report of a sor^+ isolate of a VT producing O157 from a patient with HC (Farmer & Davis, 1985). Also, prior exposure to sorbitol is known to induce sor^+ mutants among *E. coli* O157:H7. This however, does not pose a problem in the primary isolation of *E. coli* on SMAC medium.

Haldane *et al.* (1986) reported that all of their isolates of O157:H7 produced lysine and ornithine decarboxylase and that this characteristic could be used with NSF as an improved biochemical screen for *E. coli* O157:H7. A lysine negative O157:H7 has however been described (Tison, 1990).

96% of *E. coli* isolates produce the enzyme β-D-glucuronidase which cleaves the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), providing a fluorescent end product, methylumbelliferone, that is detectable under a long wave UV light source. Very few organisms other than *E. coli* are MUG positive (MUG^+). *E. coli* O157:H7 on the other hand, has been reported to be uniformly MUG negative (MUG^-), (Krishnan *et al.*, 1987; Ratnam *et al.*, 1988) and furthermore all O157 serotypes which were VT^+ were MUG^-. Thompson *et al.* (1990) developed a rapid MUG procedure (RM-MUG) to identify VT^+ strains of *E. coli* serotype O157. SMAC-MUG medium was very useful in isolating *E. coli* O157:H7 from enrichment cultures of both ground beef and dairy products. However, problems were encountered with growth of *Enterobacter*, *Proteus*, and *Hafnia* colonies with characteristics similar to those of *E. coli* O157:H7 on SMAC-MUG (i.e. sor^- & MUG^-).

A fluorescein-labeled polyclonal, affinity-purified goat antibody to *E. coli* O157:H7 has been developed that enables rapid direct immunofluorescent identification of *E. coli* O157:H7 isolated from faecal specimens on SMAC agar (Tison, 1990). Specific typing of O157:H7 can be achieved through slide agglutination using O157 and H7 antisera. A screening medium containing H7 antiserum in sorbitol fermentation medium has also been developed (Farmer & Davis, 1985) and a latex agglutination test for the rapid presumptive identification of sor$^-$ colonies from SMAC has recently been described (March & Ratnam, 1989). Another problem is that another sor$^-$ *Escherichia* species, *E. hermannii*, may agglutinate some O157 antisera (March & Ratnam, 1989). *E. hermannii* can be differentiated from *E. coli* O157:H7 by cellobiose fermentation (Ratnam *et al.*, 1988) and it has been found useful to screen samples on cellobiose-MacConkey (CMAC) medium in addition to SMAC medium.

Many other serotypes of VTEC however, have been associated with clinical disease (Ritchie *et al.*, 1992). Studies have shown that other serotypes of VTEC are sor$^+$ (Krishnan *et al.*, 1987) and therefore there is no obvious reason why the absence of sorbitol fermentation should be linked genetically to VT production. If total reliance is placed on SMAC medium there is a likelihood that serotypes other than O157:H7 will be missed. The overali diagnostic strategy, therefore must be directed towards detecting VTEC in general, rather than detecting a single serotype. At present however, there are no differential markers for non-O157 VTEC and identification must be based solely on demonstration of VT.

1.8.3 Screening VTEC for VT production and detection of free faecal VT (FVT)

Primarily, identification of strains producing different types of VT involved cytotoxicity assays and neutralisation with specific antibodies which use Vero and HeLa cell monolayers in tissue culture. A direct faecal VT assay to screen stool samples for VT1 and VT2 has been developed (Ritchie *et al.*, 1992). It involves screening stool

supernatants for verocytotoxicity and the use of a neutralisation step for confirmation of positive results. When compared with stool culture for *E. coli* O157:H7 on SMAC for the laboratory diagnosis of VTEC infections, results indicated that neither test detected all positives. Also, some specimens can be SMAC culture positive yet negative by direct FVT assay. These specimens tended to be collected early in the course of the illness and it is thought that the free toxin levels were not yet high enough to be detected in the stools. In an earlier study, Petric *et al.* (1987a) showed that addition of the protein synthesis inhibitor cycloheximide, at concentrations of less than 32 $\mu g\ ml^{-1}$, to Vero cell monolayers 1 day before the addition of VT significantly enhanced H30 VT activity in Vero cells. The reason for this is unknown. Perhaps if Ritchie and coworkers had used the enhancing effect of cycloheximide to improve the sensitivity of their Vero cell monolayers, those specimens which were negative by direct faecal assay but positive by SMAC culture might have been detected.

Whilst these bioassays are highly sensitive, they are slow, expensive, labour intensive and difficult to standardise. Maniar *et al.* (1990) has shown that the use of Vero cell suspension culture is as reliable as the use of Vero cell monolayers and provides detection of VT 24 to 48 hours earlier. However, tissue culture facilities may not be available in many clinical laboratories, especially those in underdeveloped and developing countries and therefore these assays have proved unsuitable for the screening of large numbers of bacterial isolates for epidemiological purposes.

A counter-current immunoelectrophoresis (CIE) method has been described to test for free FVT (Maniar *et al.*, 1990). This method is easy to perform and takes only two hours to complete. The test however, gave 14.4% false-negative and 6.3% false-positive results. False-positives were thought to be the result of an antibody component against the somatic antigen O157 in the antitoxin used, whereas false-negatives suggested that the minimum concentration of VT required to produce a positive result in the Vero cell system is insufficient to form a line of precipitation by the CIE test.

1.8.4 Enzyme-linked immunosorbent assay (ELISA) methods to detect VT1 and VT2

ELISA for use in diagnostic laboratories have been developed to detect many kinds of toxin such as cholera toxin, *E. coli* LT and ShT (Donohue-Rolfe *et al.*, 1986). ELISAs for detection of VTs have also been developed (Kongmuang *et al.*, 1987; Oku *et al.*, 1988; Downes *et al.*, 1989). This technique has advantages over the Vero cell assay in being more accurate, simpler, rapid and less expensive. Identification and selection of VTEC strains typically can be performed within 24 hours making ELISA suitable to test large numbers of strains or colonies efficiently and economically. At present however, these assays lack sensitivity. Picogrammes per ml (pg ml^{-1}) of toxin can be detected by the cytotoxic assay whereas nanogrammes per ml (ng ml^{-1}) of toxin were required for positive ELISA results. Only those strains producing high to moderate levels of VT can therefore be detected, whereas those strains producing low VT levels and faecal extracts containing low levels of toxin cannot.

1.8.5 Colony blot assay with VT monoclonal antibodies to detect VTEC

Attempts to develop immunochemical tests to detect toxin-producing bacteria were hampered by the presence of cross-reacting antibacterial antibodies in polyclonal antitoxin and normal sera. It was proposed therefore, that the use of MAb rather than polyclonal antisera should improve the specificity of immunochemical tests for the detection of VT. The limiting factor in the development of MAb against VT was the availability of purified toxin for immunisations, screening assays and characterisation studies. The purification of VT routinely took 3 weeks and the yield of pure toxin from an 8 litre culture was approximately 200 microgrammes (μg). Also, a major difficulty in detecting VTEC is that even for high level toxin producers the amount of VT synthesised is estimated to be less than 0.01% of total cellular protein (Karch *et al.*, 1986). Strockbine *et al.* (1985) first described the production and characterisation of

three MAb against VT. One of these antibodies to VT1 was used in the development of a colony blot ELISA to detect VTEC producing VT1, VT1 and VT2. In the development of the colony blot assay, subinhibitory concentrations of the antibiotics trimethoprim and sulfamethoxazole were incorporated into the culture media, since they dramatically increased the production of toxin by several VTEC strains, as well as ShT by *S. dysenteriae* type 1 (Karch *et al.*, 1986). At growth inhibitory doses, trimethoprim is a competitive inhibitor of dihydrofolate reductase and sulfamethoxazole is a structural analogue of paraminobenzoic acid. However, the mechanism by which trimethoprim-sulfamethoxazole stimulates toxin synthesis was not investigated. The strains tested in the colony blot assay had previously been tested for the levels of VT in the sonic lysates using the cytotoxicity assay (Marques *et al.*, 1986). When analysed with respect to the level of toxin produced, the colony blot assay appeared to be sensitive for the identification of high VT-producing strains only. VTEC strains that were classified as low to moderate or trace toxin producers were negative in the blot assay. The MAb to VT2 developed by Perera *et al.* (1988) permitted the colony ELISA of Strockbine *et al.* (1985) to be upgraded to also detect most strains of *E. coli* which produce VT2 alone. The existance of antigenic variants among the VT2 subfamily will necessitate the generation of MAb to each one to fulfill the goal of detecting all high-to-moderate level VTEC.

1.8.6 Receptor-based ELISA (RELISA) for VT's

Basta *et al.* (1989) reported a receptor-specific ELISA (RELISA) technique for the detection of VT1 based on the binding affinity of VT1 for Gb_3 (section 1.3.3.1). Lyso-Gb_3 (deacylated Gb_3) which is more polar than Gb_3 but retains VT1 binding, was used as the capture molecule. The sensitivity of this assay made it possible to detect very low levels of VT1 (down to 5 pg). The RELISA has overcome the shortfall in sensitivity by effectively concentrating the toxin from the sample for subsequent immunological detection. It is still not quite as sensitive as the cell culture cytotoxicity procedure,

which can detect purified toxin down to levels of 1 pg. The RELISA was found however, to be as sensitive as the cell culture cytotoxicity assay when used to assay VT in the culture supernatants of various VTEC strains. The assay only detected VT1 but use of anti-VT2 instead of anti-VT1 would render the assay capable of detecting VT2 and a mixture of both antibodies would detect both toxins. Another RELISA has been developed using P1 glycoprotein from hyatid cysts (Acheson *et al.*, 1990). This assay detects ShT, VT1 and VT2 and is sensitive in the subnanogramme level.

Even with these sensitive tests there are some cases where VTEC cannot be isolated and no faecal VT found. An alternative test in this situation is to demonstrate a serum antibody response to VT. A modification of the RELISA developed by Basta *et al.* (1989) has recently been described (Boulanger *et al.*, 1990) for the detection of NAb to VT1 (NEUTRELISA). This assay can be performed in 6 hours as opposed to the cytotoxicity neutralisation assay, which requires up to three days. The NEUTRELISA will be useful in the diagnosis of HUS, particularly for those patients who present late in their illnesses and whose stools do not demonstrate VT. Future modifications of the RELISA technology are expected to result in the development of rapid assays for the detection of VT2 and its NAb. Application of such a battery of assays would provide comprehensive seroepidemiologic data on VTEC infections.

1.8.7 Immunoassay detection methods for VTEC from food

Isolation and detection methods first used for detection of VTEC in foods concentrated on the isolation of *E. coli* O157:H7 by plating foods on selective media modified to allow the growth of O157 strains (incubation temperature less than 45^{0}C), and to demonstrate their typical biochemical reactions (inability to ferment sorbitol, lack of fluorescence in the presence of MUG and production of indole). Doyle & Schoeni (1987) have used a hydrophobic grid membrane filter (HGMF) immunoblot technique and PAb raised against a crude VT preparation to identify all VT1 and VT2 producing

bacterial strains in retail meats. Todd *et al.* (1988) later used HGMF to isolate and enumerate *E. coli* O157 from food with specific identification by an anti-O157 MAb. They found that use of a MAb rather than a less specific polyclonal avoided cross-reaction with non-O157 strains that had yielded false positives in other identification schemes (e.g *E. hermannii*). Group N *Salmonellae* however could not be excluded and required further investigation with antisera to identify any group N *Salmonella* spp. present. The sensitivity of this HGMF method was 10 *E. coli* per gramme (g) which was not as great as the more laborious research enrichment method of Doyle & Schoeni (1987), (1.5 *E. coli* O157:H7 per g). Recently a MAb raised using a rough strain of *E. coli* O157:H7 has been produced that is specific for *E. coli* O157:H7 and O26:H11 (Padhye & Doyle, 1991a). This MAb reacted specifically with two chromosomally encoded outer membrane proteins of apparent MW of 5000 to 6000 which appeared to be markers specific for EHEC strains of serotypes O157:H7 and O26:H11. This MAb, because of its specificity has been now used in an enrichment-sandwich ELISA for the rapid and sensitive detection, (0.2-0.9 *E. coli* O157:H7 per g), of these types of EHEC isolates in food (Padhye & Doyle, 1991b).

1.8.8 VT1- and VT2- specific DNA probes

DNA or RNA hybridisation with probes derived from virulence genes has previously been demonstrated to be an alternative technique which can facilitate detection of bacteria containing similar pathogenic characteristics (Moseley *et al.*, 1980).

A DNA probe for the detection of a 60 MDa plasmid typically carried by O157:H7, that promotes epithelial cell attachment of VTEC has been prepared by Levine *et al.* (1987). However, because plasmids of *E. coli* may be lost during isolation, this probe would not detect EHEC isolates that no longer carry the 60 MDa plasmid. Specific DNA probes have been developed to identify DNA sequences that code for VT1 and VT2. These probes have advantages in that the hybridisation test is specific and sensitive and that a

large number of test samples can be processed at one time. In addition gene probing is considerably faster than, and probably as specific as testing culture supernatants or sonic lysates in tissue cultures for cytotoxicity and inhibiting their effect with antibodies to VT1 and VT2. DNA probes however, do not give an indication of the level of toxin expression, which has been shown to be important in studies of pathogenesis (Marques *et al.*, 1986).

1.8.8.1 Cloned DNA fragments as probes

Willshaw *et al.* (1987) reported identifying restriction fragments from VT1 and VT2 clones that may be purified and used as VT gene probes. Newland & Neill (1988) also developed two pairs of DNA probes to identify and distinguish those strains which encode VT1 and VT2. The first pair consisted of internal restriction fragments cloned from the structural genes for VT1 and VT2. The VT2 probe in this study also functions as a probe for VTe because the VT2 fragment was chosen from a region of the VT2 gene which contains sequences homologous to those present in VTe. These cloned DNA probes however, are not able to differentiate between the genes coding for VT2 and VT2v. The second pair of probes consisted of restriction fragments not associated with VT synthesis and are used to assess the association of phage type with VT type.

DNA probes have been used to detect VTEC in food and calf faecal samples (Samadpour *et al.*, 1990). Enrichment cultures prepared from food or faecal samples were probed for VTEC by colony hybridisation or by dot blot. VTEC were detected at concentrations as low as 1.3 colony forming units (CFU) per g of sample. The dot blot technique yielded results within 48 hours, whereas the colony hybridisation technique takes 3 to 4 days but permits recovery of the positive colonies when desired. Gene probing can be performed with ease and sensitivity to the screening of food and environmental samples for the detection of organisms bearing the VT genes. This technique has two definite advantages. First of all VTEC, regardless of serotype, can be

detected and recovered and cross-reactive organisms that could be detected by the antibodies to the O157 antigen are avoided. Also after hybridisation and exposure to X-ray film the colony hybridisation and dot-blot filters can be washed and reprobed. This makes the method suitable for detection of a wide range of pathogens for which specific DNA probes are available and which would grow well in the enrichment media.

Gene probes are a useful diagnostic technique but are not available to most clinical laboratories due to the cost of reagents with short shelf lives, concerns of handling and disposal of radionucleotide-labeled DNA. Gicquelais *et al.* (1990) describe a simple and economical method for using biotin-labeled DNA probes with bacterial colony blots to identify diarrhoea-causing *E. coli*. The biotinylated probe method exploits the high affinity of streptavidin for biotin-labeled molecules in a sandwich system analogous to an indirect ELISA. A streptavidin-alkaline phosphatase conjugate is used to colorimetrically detect biotin-labeled DNA probes which have hybridised to target DNA present on the filter paper. Biotinylated DNA probes as well as their ease and safety in handling have a long shelf life. Laboratories with limited budgets and those that do not have the facilities to use radioisotopes will benefit from this technique.

1.8.8.2 Synthetic oligonucleotide probes

Analysis of the nucleotide sequences of VT1 (Jackson *et al.*, 1987a) and VT2 (Jackson *et al.*, 1987b) suggested that oligonucleotide probes could be constructed and used to identify VTEC. The structural genes of VT1 and VT2 are 58% homologous in overall nucleotide sequence, although there are localised regions of much lower or much higher homology. A sequence of high homology might be used as a probe to detect *E. coli* producing VT1, VT2 and VTe, while a region of low homology could be used as a probe to differentiate between *E. coli* producing different forms of VT. A number of oligonucleotide probes have been synthesised to enable detection of the different types of VT by DNA hybridisation. Details of these are shown in table 1.7.

Table 1.7 **Synthetic oligonucleotide probes for VT DNA hybridisation**

Oligo-nucleotide probe	Toxin type detected	Oligonucleotide sequence (5'-3')	Location within gene[a]	Reference
772[a]	VT1	GATGATCTCAGTGGGCGTTC	772-791	Karch & Meyer (1989a)
849[b]	VT2, VTe	TCTGAAACTGCTCCTGTGTA	849-868	
428-I[b]	VT1	GATACTGGCTCAGGGCATAA	428-447	Karch & Meyer (1989b)
428-II[b]	VT2, VT2v	AACCACACCCACGGCAGTTA	428-447	
I[a]	VT1	ACTGCAAAGACCTATGTAGATTCGCTGAATGTCATTCGCTC	250-290	Meyer et al. (1989)
II[a]	VT1	TGATCTCAGTGGGCGTTCTT	774-793	
III[a]	VT1	CTTACATTGAACTGGGGAAGGTTGAGTAGCGTCCTGCCTGA	820-860	
IV[a]	VT1	ACCTTTACAGTTAAAGTGGGTGATAAAGAATTATTTACCAA	1231-1271	
V[b]	VT2, VTe	TCTGAAACTGCTCCTGTGTATACGATGACG	849-878	
AI[b]	VT1	ATACTGAATTGTCATCATCA	1013-1032	Brown et al. (1989b)
BII[b]	VT2, VTe	AAGTATAATGAGGATGAEACATTTACAGTG	1297-1326	
E5135[b]	VT2	CCCGTCAACCTTCACTGTAAATGTGTC	1312-1338	Nil et al. (1991).
A8157[c,d]	VT2v	GATACATTCACAGTAAAAGTGGCCGGA	1312-1338	

[a] DNA sequences of genes according to De Grandis et al. (1987).

[b] DNA sequences of genes according to Jackson et al. (1987b)

[c] Oligonucleotide A8157 has the sequence of the sense (+) strand, while E5135 has the sequence of the antisense (-) strand.

[d] Sequence of A8157 is based on the sequence derived from Nil et al. (1991).

[e] Location corresponds to the position of bases in the previously published nucleotide sequences of the slt-I[a] and slt-II[b] genes.

1.8.8.3 Synthetic oligonucleotide primers

DNA hybridisation with specific polynucleotide (Willshaw *et al.*, 1987; Newland & Neill, 1988) and oligonucleotide (table 1.7) probes complementary to VT gene sequences has been shown to facilitate detection of VT producers. However, several problems must be faced before standard immunological or DNA hybridisation detection methods for VTEC can be introduced for routine laboratory use. One of these difficulties is serological and genetic heterogeneity among VT's. In the VT operons sequenced to date there are conserved regions especially in the genes that encode A subunits (Karch & Meyer, 1989b). If these regions are several hundred base pairs apart from each other they are suitable targets for oligonucleotides used as primers to amplify the DNA segment between these nucleotide sequences by PCR. Different sets of oligonucleotide primers have been devised and used to detect the different types of VT by PCR. Base sequences, locations and predicted sizes of amplified products for these VT-specific oligonucleotide primers are shown in table 1.8.

Karch & Meyer (1989b) produced a single pair of oligonucleotide primers designed to amplify a segment of the VT genes by PCR which were nonidentical to sequences published for genes for VT1 and VT2. These so-called degenerated primers contained intentionally introduced sequence ambiguities from highly conserved regions among genes for VT's to efficiently amplify sequences from VTEC strains irrespective of the type of toxin produced. Furthermore they synthesised two additional oligonucleotides with the capacity to determine whether the amplified segments are derived from VT1 or VT2 genes. Pollard *et al.* (1990a) designed a set of four synthetic oligonucleotide probes derived from the sequences of the VT1 and VT2 genes and used these in a PCR amplification procedure to detect these genes in some enteric pathogens. The oligonucleotide probes clearly distinguished VT1 and VT2 strains of *E. coli* and did not give specific amplification with nucleic acid from VTe-producing *E. coli*. Johnson *et al.* (1990) further synthesised a set of oligonucleotide primers specific for the VTe A

Table 1.8 Base sequences, locations and predicted sizes of amplified products for VT-specific oligonucleotide primers

Primer	Toxin type (5'-3')	Oligonucleotide sequence sequence[a1-5]	Location within gene	Size and location of amplified product (bp)	Reference
MK1	ShT, VT1, VT2,	TTTACGATAGACTTCTCCGAC[b]	311-330	227 (VT1A)[c]	Karch & Meyer (1989b)
MK2	VT2v, VTe	CACATATAAATTATTTCGCTC[b]	515-535	224 (VT2A)	
VT1a	ShT, VT1	GAAGAGTCCGTGGGATTACG[a1]	1191-1210	130 (VT1B)	Pollard et al. (1990a)
VT1b		AGCGATGCAGCTATTAATAA[a1]	1301-1320		
VT2a	VT2	TTAACCACACCCACGGGAGT[a3]	426-445	346 (VT2A)	
VT2b		GCTCTGGATGCATCTCTGGT[a3]	752-771		
VT1c	VT1	ACCCTGTAACGAAGTTTGCC[a2]	31-50	140 (VT1 promoter)	Pollard et al. (1990b)
VT1d		ATCTCATGCGACTACTTGAC[a2]	151-170		
VTe-a	VTe	CCTTAACTAAAAGGAATATA[a4]	217-236	230[d]	Johnson et al. (1990)
VTe-b		CTGGTGGTGTATGATTAATA[a4]	427-446		
VT2-c	VT2, VT2va & b	AAGAAGATGTTTATGGCGG1[a3]	1210-1229[e]:1213-1232[f]	285	Tyler et al. (1991)
VT2-d		CACGAATCAGGTTATGCCTC[a3]	1475-1494[e]:1478-1497[f]		
VT2v-1	VT2va & b	CATTCACAGTAAAAGTGGCC[a5]	1319-1338[f]	385	
VT2v-2		GGGTGCCTCCCGGTGAGTTC[a5]	1684-1703[f]		
stx/sltI 5'	ShT, VT1	GACAGGATTTGTTAACAGG[a2]	460-478	680 (VT1A/ShTA)	Jackson (1991)
stx/sltI 3'		TTCCAGTTACACAATCAGGC[a2]			

a Primers designed on published DNA sequences for VT1 (Calderwood et al., 1987[1] ; Strockbine et al., 1988[2]), VT2 (Jackson et al., 1987b[3]), VTe (Weinstein et al., 1988a[4]), VT2v (Ito et al., 1990[5]).

b Primers MK1 and MK2 are nonidentical to the gene sequences for VT1 and VT2 but contain intentionally introduced ambiguities such that they show at least 90% homology to their target sites for both VT1A and VT2A.

c VT1A gene segment is longer by 3 bases because it contains an additional triplet at position 449 that is not present in the VT2A subunit gene.

d Encompasses regions for the proposed promoter and a portion of the A subunit of the VTe operon.

e,f Encompasses regions for VT2[e] and VT2va & b[f] B subunits respectively.

subunit gene which when used in association with the primers for VT2 (Pollard *et al.*, 1990a), will rapidly and specifically distinguish VT2 and VTe genes in VTEC. Although VT2 and VTe share extensive DNA sequence homology they can be differentiated and clearly distinguished by varying the degrees of stringency in the PCR protocol. Use of these PCR protocols allowed the definitive characterisation of strains originally suspected to be human VTe isolates because of their low cytotoxicity to HeLa cells, as VT2 and not VTe producers. None of the biological, immunological or other molecular techniques described previously could differentiate ShT from VT1. Although it is impossible to distinguish the gene products, a unique sequence in the promoter region of the *slt*-I gene has allowed genetic differentiation of VT1 from ShT using PCR technology (Pollard *et al.*, 1990b). As mentioned in section 1.4.3.4, Tyler *et al.* (1991) designed a set of synthetic oligonucleotide primers for use in the PCR protocol that can specifically detect the B subunit genes in *vtx*2ha and *vtx*2hb. An additional set of primers amplified a fragment common to the B subunits of the VT2 and VT2v genes. Subsequent restriction endonuclease digestion of this amplicon permitted prediction of specific VT2 and variant genotypes on the basis of predetermined restriction fragment length polymorphisms.

The PCR technique is relatively simple and combined with its superior sensitivity and specificity should enable rapid screening for the presence of VTEC in clinical specimens and food samples. The ability to analyse specimens without the isolation of specific organisms should facilitate rapid diagnosis and treatment. Subsequent to extraction of DNA, the PCR protocol yields in 4 hours, data that would require several days by traditional tissue culture assays. Furthermore, as no correlation exists between the intensity of amplification products observed in the PCR and cytotoxin titres, the application of the VT1-specific PCR protocol (Pollard *et al.*, 1990b) to a larger group of *E. coli* isolates should clarify the significance of low-level VT1 production previously reported in some strains. However, because these PCR methods may require purified DNA, visualisation of the amplified product by agarose gel electrophoresis, or Southern

blot hybridisation with a radioactive DNA probe, they are not entirely appropriate for screening large numbers of samples in diagnostic laboratories. Olive (1989) adapted the PCR technique for the identification of ETEC strains and used an alkaline phosphatase (PhoA)-labeled DNA probe in a spot blot assay to detect amplified products, making it better suited to clinical laboratories. More suited to rapidly screening large numbers of clinical isolates, Jackson (1991) describes a PCR technique to detect ShT and VT1 in which individual colonies from solid medium were inoculated directly into the PCR mixture. The PCR products from liberated target DNA were amplified with *stxA*/*slt*I gene primers and labeled by direct incorporation of the non-radioactive TTP analogue, digoxigenin-11-dUTP (DIG) in the reaction mixture. DIG-labeled PCR products were then hybridised to homologous DNA immobilised on a nitrocellulose membrane and detected immunologically by using an PhoA-conjugated antibody to digoxigenin. In future studies, the feasibility of this non-radioactive PCR detection method may be assessed using additional primer pairs capable of detecting strains which produce other VT types.

1.9 Vaccines to VTs

A toxoid form of whole ShT has been used in the past as an immunogen and shown to produce a protective antibody response to ShT in rabbits (Donohue-Rolfe *et al.*, 1984). Specifc antibodies to the B subunit were affinity purified from this serum and shown to neutralise the cytotoxic activity of ShT towards HeLa cells (Donohue-Rolfe *et al.*, 1984). Boyd *et al.* (1991) developed a vaccine candidate against the effects of ShT and VT1 in humans by immunising rabbits with VT1 B subunit. They avoided the issue of residual toxicity this way and obtained sera with high neutralising titres against VT1. No neutralising activity against VT2 was observed in the Vero cell assay. In an attempt to develop an effective vaccine against ED, Gordon *et al.* (1992), using oligonucleotide-specific site-directed mutagenesis have identified an amino acid in the A subunit that drastically reduces the enzymatic activity of VTe. Pigs vaccinated with this mutant

toxin, designated SLT-IIvE167Q, developed a NAb titre of 1:512, 21 days postinjection and their tissues were free of ED lesions. These data suggest that SLT-IIvE167Q may represent an effective vaccine against ED. As yet, no potential vaccine candidates have been described for use in immunisation against the potentially fatal VTEC associated syndromes of HC and HUS.

1.10 Aims of the project

A pathogen is often defined as a microorganism that has the capacity to cause disease in a particular host (Falkow, 1990) and not unexpectedly, a good deal of research effort has been and is, directed at the treatment and prevention of these diseases. Ever since bacteria were first recognised as causing diseases, investigators have been intent on understanding how they do so and major advances, fueled largely by application of a genetic approach, have been made in the biochemistry, immunology and cell biology of the host-parasite interaction. For many years the only bacteria that could be studied at the molecular level were those in which one or more genetic systems for the transfer of DNA were available. This allowed the mapping of a few bacterial genomes and the identification of genes whose products regulated other genes *in trans*. The advent in the last decade of recombinant DNA and gene cloning techniques, as well as rapid DNA sequencing methods has resulted in an explosion of research on the molecular mechanisms of regulation of virulence determinants in bacterial pathogens.

Microbial pathogenesis is usually complex and multifunctional. The host microenvironment varies considerably during the course of infection and a successful pathogen must be capable of regulating many different factors both coordinately and sequentially to prevent the host from eliminating it and to enable it to grow and multiply. Study of this phenotypic modulation of virulence gene expression in response to environmental stimuli converts the extraordinarily complex host-parasite relationship to the more tractable task of studying bacterial physiology and can offer insights into the strategies of pathogenesis.

There is considerable clinical, genomic and pathogenic information relating to VT's, but little pertinent to the physiological state of the bacterium. This project was initiated to examine the physiology of VT production by *E. coli*. Control of expression of genes encoding virulence determinants is usually at the level of transcription (Mekalanos,

1992). Consequently it was necessary to develop an assay to enable quantification of VT1 and VT2 gene expression at the molecular level, as no rapid and specific assay of this type was available at the time. Subsequently this assay could be used to attempt to elucidate the relationship between the various nutritional and physical parameters that control VT1 and VT2 synthesis in laboratory culture and then correlate these with potential signals that may trigger toxin synthesis during the infection cycle.

CHAPTER TWO

2 Materials and methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in tables 2.1 and 2.2 respectively.

Table 2.2 Plasmids

Plasmid	Phenotype	Source	Reference
NTP705	Kn^r *slt*-IB'	B. Rowe	Willshaw *et al.* (1985)
NTP707	Tc^r *slt*-II	B. Rowe	Willshaw *et al.* (1987)
pSC105	Ap^r Kn^r, *slt*-IA'::Tn*phoA*	S. Calderwood	Calderwood & Mekalanos (1987)
pSLF22	Kn^r, *slt*-II::Tn*phoA*	This study	
pSLF34	Kn^r, *slt*-II::Tn*phoA*	This study	

2.2 Bacteriophage

The bacteriophage used in this study are listed in table 2.3.

Table 2.3 Bacteriophage

Phage	Source	Reference
Lambda *Vir*	P. Reeves, Warwick University	-
Lambda Tn*phoA*	P. Reeves, Warwick University	Manoil & Beckwith (1985)

Table 2.1 Bacterial strains

Strain	Genotype	Source	Reference
E. coli O157:H7	-	ATCC 35150	Wells (1983)
E. coli O26:H11	-	NCTC 8781	Parker, M.T. (1953). PHLS Manchester
E. coli O157:H7 Clinical isolate	-	Warwick Hospital	-
E. coli K12	-	Warwick University	-
E. coli O26:H11 (E3787/H19)	-	S. Scotland	Willshaw *et al.* (1987)
E. coli O157:H$^-$ (E32511)	-	S. Scotland	Willshaw *et al.* (1987)
E. coli C600(933J)	-	T. Meyer	Meyer *et al.* (1989)
E. coli C600(933W)	-	T. Meyer	Meyer *et al.* (1989)
E. coli K12 6OR746	-	B. Rowe	Willshaw *et al.* (1985)
E. coli K12 6OR363	-	B. Rowe	Willshaw *et al.* (1987)
E. coli DH1	F$^-$, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, ($r_k^-M_k^+$), *sup*E44,λ^-	P. Reeves	Hanahan (1983)
E. coli CC118	F$^-$, (*ara-leu*)7697 *ara*D139, (*lac*)X74, *pho*A 20, *gal*E, *gal*K, *thi*, *rps*E, *rpo*B, *arg*E(Am), *rec*A1	P. Reeves	Manoil & Beckwith (1985)
E. coli TG1	(*pro-lac*),^{s}*sup*E, *thi*, F$^-$, *tra*O, *pro*AB_5^+, *lac*I^q, *lac*Z$^{\Delta}$15	D. Cardy	Carter *et al.* (1985)
S. dysenteriae Type 1	-	S. Colby	-

2.3 Media

All media, unless otherwise stated, was sterilised by autoclaving at 121°C for 15 minutes (min). Solid media was prepared by the addition of 1.5% weight by volume (w/v) Difco Bacto Agar. Soft media was prepared by the addition of 0.5% (w/v) Difco Bacto Agar. For use as top agar in the preparation of bacterial lawns (2.11.2), the agar was remelted and kept at 50°C in a water bath.

2.3.1 DDA

	per litre
Bacto tryptone	20 g
NaCl	8 g

After sterilisation and once cool, $MgSO_4$ was added to 10 mM from 1 M stock.

2.3.2 LB (Luria Bertani)

	per litre
Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g
	(pH 7.5)

2.3.3 Iron limited media

For growth under low iron conditions, LB media was supplemented with 0.2 mM 2,2'-dipyridyl (Sigma) from a 0.1 M 50% volume by volume (v/v) ethanol:water stock, as described by Calderwood & Mekalanos (1987).

2.3.4 LB/Mal/Mg^{2+} media

To sterile LB was added: Maltose to 0.2% (w/v) from 20% (w/v) stock and Mg^{2+} to 10^{-2} M from 1 M $MgSO_4$ stock.

2.3.5 Low phosphate media. (Roeder & Collmer, 1985)

	per litre
$MgSO_4.7H_2O$	0.1 g
$(NH_4)_2SO_4$	1.0 g
Na citrate	0.5 g
Tris	12.1 g
	(pH 7.4)

After sterilisation and once cool, KH_2PO_4 was added to 100 μM from 100 mM stock and glucose to 0.2% (w/v) from 20% (w/v) stock.

2.3.6 Minimal salts media

	per litre
Minimal Salts (x4)	250 ml
20% (w/v) Glucose	10 ml

Minimal salts solution (x4).

NH_4Cl	20.0 g
NH_4NO_3	4.0 g
Na_2SO_4 anhydrous	8.0 g
K_2HPO_4 anhydrous	12.0 g
KH_2PO_4	4.0 g
$MgSO_4.7H_2O$	0.4 g

Each salt was added in the order indicated to 1 litre of distilled water (DW).

2.3.7 M9 minimal media

	per litre
M9 salts (x10)	100 ml
$CaCl_2$ 10 mM	10 ml
$MgSO_4$	0.1 M

M9 salts (x10)

	per litre
Na_2HPO_4 anhydrous	60 g
KH_2PO_4 anhydrous	30 g
NH_4Cl	10 g
NaCl	5 g
	(pH 7.4)

2.3.8 Penassay media

Penassay broth (Difco Antibiotic Medium No.3) was prepared according to the manufacturer's instructions.

2.3.9 TYE

	per litre
Bacto tryptone	10 g
Bacto yeast extract	10 g
NaCl	5 g

2.4 Antibiotics

Antibiotic stock solutions were prepared as described in Maniatis *et al.* (1982) and used at the following concentrations (unless otherwise stated).

Antibiotic	Stock concentration ($mg\ ml^{-1}$)	Final concentration ($\mu g\ ml^{-1}$)
Ampicillin (Ap)	100	100
Chloramphenicol (Cm)	35	10
Kanamycin (Kn)	25	50
Tetracycline (Tc)	12.5	15

2.5 General chemicals and materials used in this study

Chemicals	Supplier
^{32}P-dCTP	Amersham
DNA sequencing nucleotides	Pharmacia
DNA sequencing materials (acrylamide, bisacrylamide, AMPS, SDS and urea)	BioRad Labs
General materials	BDH/Sigma or Fisons
^{35}S-methionine	Amersham
Nitrocellulose filters (Hybond-C)	Amersham
Organic acids and Solvents	May & Baker, Fisons or BDH
Restriction enzymes and buffers	Amersham or BRL
X-ray film	Fuji Photo Film Co. Ltd.

2.6 General solutions/buffers

These solutions are referred to in the text of this chapter. Their compositions and where appropriate, the procedures for their preparation are described below.

Aniline reagent	A universal vial (calibrated to 11 ml) was washed with concentrated HCl, rinsed thoroughly with sterile DW (SDW) and dried in an oven. 0.5 ml glacial acetic acid, 7 ml SDW and 1 ml redistilled aniline were added and the pH adjusted to 4.5 with glacial acetic acid. This solution was stored in the dark at 4^{o}C.

Chloroform (for DNA extraction)	24:1 (v/v) chloroform:isoamyl alcohol
Denhardts solution (x50)	5 g Ficoll 5 g Polyvinylpyrrolidone BSA (Pentax fraction V) DW to 500 ml
ENDO buffer (x2)	25 mM Tris-HCl pH 7.6 25 mM KCl 5 mM $MgCl_2$
Formaldehyde loading buffer (FLB)	50% (v/v) Formamide 6% (v/v) Formaldehyde 1x MOPS Buffer 10% (v/v) Glycerol 100 $\mu g\ ml^{-1}$ Ethidium Bromide 0.025% (w/v) Xylene Cyanol 0.025% (w/v) Bromophenol Blue
Formamide (for ricin assay)	deionised by addition of 1 g of Duolite MB6113 (BDH) resin to 100 ml formamide. This was stirred for 1 hour, filtered through Whatman No.1 filter paper and 0.5 M NaOH added to pH 7.0. This was stored in the dark at 4^oC.
Formamide (for RNA extraction)	deionised by addition of 5 g mixed bed resin (Bio-Rad AG501-X8, 20-50 mesh) to 50 ml formamide. This solution was stirred for 30 min, filtered through Whatman No.1 filter paper and stored in aliquots at -20^oC.
Kinase buffer (x10)	700 mM Tris-HCl pH 8.0 100 mM $MgCl_2$
Loading buffer type IV (x6)	0.25% (w/v) Bromophenol Blue 40% (w/v) Sucrose

Loading buffer (x5) (for Western Blotting)	0.125 M Tris-HCl pH 6.8 10% (w/v) Sucrose 4% (w/v) SDS 0.25% (w/v) Bromophenol Blue
Lysis mix	0.05 M Tris 0.0625 M EDTA 2% (w/v) Bri j58 (Sigma) 0.4% (w/v) Na deoxycholate
MOPS buffer (x5)	5 mM EDTA pH 8.0 50 mM Na Acetate 200 mM MOPS pH 7.0
Phage buffer	10 mM Tris pH 7.4 10 mM $MgSO_4$ 0.01% (w/v) Gelatin
Phenol (for DNA extraction)	Neutralised with 1 M Tris-HCl pH 8.0, and containing 0.1% (w/v) 8-hydroxyquinoline
Phenol (for RNA extraction)	100 g BRL ultra pure phenol melted at 68^{o}C. 0.1 g 8-hydroxyquinoline was added and the mixture left overnight to equilibrate in 20 mM Na Acetate buffer, pH 5.6
Potassium phosphate buffer (pH 7.0)	30.5 ml Na_2HPO_4 (0.2 M) 19.5 ml $NaH_2PO_4.2H_2O$ (0.2 M) To 100 ml with SDW
SAE	0.5% (w/v) SDS 1 mM EDTA pH 8.0 20 mM Na Acetate pH 5.6

Salmon sperm DNA (denatured)	The DNA (Type-III sodium salt, Sigma) was dissolved in water (10 mg ml^{-1}) and sheared by passing several times through an 18-gauge hypodermic needle. After boiling for 10 min aliquots were stored at -20°C.
Sequencing buffer (x5)	200 mM Tris-HCl pH 7.5 50 mM $MgCl_2$ 250 mM NaCl
Sodium acetate (3 M pH 6.3)	3 M Na Acetate brought to pH 6.3 with acetic acid.
SSC (x20)	per litre 175.3 g NaCl 88.2 g Trisodium citrate pH 7.0
SSPE (x20)	per litre 174 g NaCl 27.6 g $NaH_2PO_4.H_2O$ 7.4 g EDTA pH 7.4
STET	8% (w/v) Sucrose 50 mM Tris-HCl pH 8.0 50 mM EDTA 5% (w/v) Triton X-100
Sucrose solution	36 mM Tris-HCl pH 7.8 40% (w/v) Sucrose 2 mM EDTA
Tris borate (TBE)	0.089 M Tris-borate 0.089 M Boric acid 0.002 M EDTA

TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0
Tris-phosphate (TPE)	3.6 mM Tris 3 mM NaH_2PO_4 0.2 mM EDTA
Tris-sucrose (for DNA extraction)	0.05 M Tris 25% (w/v) Sucrose pH 8.0
Western transfer buffer (WTB)	per litre 14 g Glycine 2.42 g Tris 200 ml Methanol

2.7 Growth and maintenance of bacterial cultures

2.7.1 Organism maintenance

All strains (with the exception of *E.coli* TG1) were maintained on LB agar plates containing the appropriate antibiotics where required, for up to 6 weeks at $4^{o}C$. *E. coli* TG1 was maintained on M9 minimal agar plates containing 0.1 ml, 0.1% (w/v) thiamine per litre. For longer term storage, an overnight culture was mixed with an equal volume of sterile glycerol in a glass vial and stored at $-20^{o}C$ and $-70^{o}C$.

2.7.2 Growth conditions

Strains were routinely grown at $37^{o}C$ in a Gallenkemp Orbital Shaker (250 rpm). Liquid cultures were propagated overnight by inoculating 10 ml LB (plus appropriate

antibiotics) in 25 ml universal bottles, with a single colony from an agar plate. A variety of glass culture vessels were employed with capacities ranging from 25 ml to 2 litres.

2.7.3 Spectrophotometry

Routine determinations of culture optical density (OD) were performed at a wavelength of 600 nanometres (nm) through a 1 cm light path cell using a Shimadzu UV-150-02 double beam spectrophotometer.

2.7.4 Viable count determinations

Routine determinations of culture viability were performed by plating 100 μl of serial dilutions in sterile phosphate buffered saline (PBS) onto the appropriate agar plates. After overnight incubation at 37^{o}C, colony counts were made and the colony forming units per ml (CFU ml^{-1}) determined.

2.7.5 Cell size distribution analysis by Coulter Counter

A Coulter Counter model 256 and Coulter Counter Channelyser C1000 connected to a BBC microcomputer were used to determine size distribution and cell counts. Samples were diluted in 20 ml balanced salt electrolyte (Isoton-Coulter Electronics Ltd.) and the total particle count and cell size distribution profiles obtained using a 30 μm orifice probe. Subtraction of background count (isoton alone) enabled the corrected count for the samples to be calculated. Latex particles of known size were used as calibration standards for cell volume analysis and the mean volume of particles (μm^3), estimated from the size distribution profiles.

2.8 API20E profiles

The API20E system (Bio Mérieux, France) is a standardised, miniaturised version of conventional procedures for the identification of Enterobacteriaceae and other Gram negative bacteria. A single colony of each strain to be tested was resuspended in 5 ml of SDW and used to inoculate the API20E strips, which were then incubated at 37°C for 18-24 hours. The addition of reagents and the interpretation of reactions were done according to the manufacturer's directions. The pattern of reactions was then coded into a numerical profile and identification made with the computer based API Profile Recognition System.

2.9 Serological identification of *E. coli* O157:H7

A latex agglutination test (Oxoid Diagnostic Reagents) was used as described by the manufacturer to determine whether the *E.coli* Clinical Isolate (table 2.1) belonged to the O157 serogroup. In addition, 10 μl of a single colony of the Clinical Isolate, resuspended in 100 μl of sterile PBS, was mixed on a slide by tilting with 10 μl of *E. coli* H7 antiserum (Difco). Agglutination with the latter was observed when small clumps of cells became visible. In both tests a negative control of *E. coli* K12 and positive control of *E. coli* O157:H7 (ATCC 35150) were used to provide comparisons.

2.10 Determination of auxotrophic requirements

Amino acid, vitamin and purine/pyrimidine pools were used in the determination of auxotrophic requirements as described by Clowes & Hayes (1968).

2.11 Phage production and phage challenge

2.11.1 Preparation of phage lysates

Exponential cultures were grown in LB/Mal/Mg^{2+} media (section 2.3.4). Mitomycin C (Sigma) was then added to 1 μg ml^{-1} from 1 mg ml^{-1} stock and cultures were further incubated overnight. Cultures were pelleted in an MSE Chilspin (5,280g, 10 min) and the supernatant removed. A few drops of chloroform were added, the supernatant vortexed, pelleted and stored at $4^{o}C$.

2.11.2 Preparation of lawns and phage challenge

The strain to be used as a lawn was grown overnight in 10 ml LB/Mal/Mg^{2+}. 0.2 ml was then added to 3 ml LB/Mal/Mg^{2+} top agar, gently mixed and poured onto a fresh LB/Mal/Mg^{2+} plate. 10 μl of the phage lysate to be tested was spotted onto the prepared lawn, the plates incubated at $37^{o}C$ overnight and examined for evidence of cell lysis/plaque production, indicative of phage.

2.11.3 Preparation of high titre lambda *vir* lysate

E. coli LE392 was grown overnight in 10 ml LB/Mal/Mg^{2+}. 200 μl volumes were then aliquoted into sterile bijouxs and 10 μl of a range of dilutions of lambda *vir* (table 2.3) in phage buffer (section 2.6) added. 4 ml LB/Mal/Mg^{2+} soft agar was added, gently mixed and poured onto solid LB/Mal/Mg^{2+} plates which were incubated overnight at $37^{o}C$. Two individual plaques were removed with a glass pipette and resuspended in 1 ml of phage buffer. 10, 50 and 100 μl aliquots were mixed with 200 μl of a fresh LE392 overnight culture, added to 3 ml soft DDA (section 2.3.1) and overlayed onto fresh DDA plates. After overnight incubation, phage were harvested from the plate which showed confluent lysis by scraping off the top agar with a sterile glass loop into a sterile

universal. The plate was washed with 2 ml phage buffer and the washings added to the top agar. 0.5 ml chloroform was added and the mixture vortexed until a soft slurry had formed. This was then pelleted in an MSE Chilspin (5,280g, 10 min) and the supernatant collected into a sterile universal. A further 0.5 ml chloroform was added and the lysate stored at $4^{o}C$.

2.11.4 Phage titre determination

The phage titre was determined by spotting 10 μl of a range of dilutions of phage lysate in phage buffer onto a fresh lawn of *E. coli* LE392. After incubation at $37^{o}C$ overnight the number of plaques were counted and the plaque forming units per ml (PFU ml^{-1}) determined.

2.12 Chromosomal DNA extraction

(a) Method 1

Chromosomal DNA extraction was carried out using the technique described by Alley (1987) with the following modifications. A 500 ml overnight culture grown on Penassay was harvested by centrifugation in a Beckman 6x250 rotor (22,100g, 10 min, at $4^{o}C$). The pellet was resuspended in 10 ml of Tris-sucrose and once resuspended, 5 ml of TE plus 150 mg of lysozyme (Sigma) were added. After incubation at $37^{o}C$ for 10 min, 15 ml of 0.25 M EDTA pH 8.0 was added and the cells incubated for a further 50 min at $37^{o}C$. Sarkosyl was then added to a final concentration of 2% (w/v) and the mixture incubated for a further hour at $50^{o}C$. It was found unnecessary to add proteinase K (BCL) to improve lysis at this point. 30 g of caesium chloride (CsCl) were added and dissolved before adding 1 ml of 10 mg ml^{-1} ethidium bromide. The resulting mixture was centrifuged in a Beckman 8x50 rotor (27,200g, 15 min, at $15^{o}C$) to remove cell debris. The supernatant was decanted, placed in a Vti50 self-seal tube and spun in a

Beckman L8-70 (196,400g, 16 hours, 20°C). The DNA in the middle of the gradient was removed (approximately 5 ml) using a hypodermic needle and extracted 3 times with water saturated butanol (to remove ethidium bromide). 3 M Na Acetate pH 6.3 (2 ml), 1 % (w/v) sarkosyl (2 ml), TE (11 ml) and isopropanol (10 ml) were added to the 5ml DNA solution. This was placed at -20°C for 2 hours and centrifuged in a Beckman 8x50 rotor (39,200g, 10 min, 4°C). The DNA pellet was washed with 70% (v/v) ethanol and dried under vacuum. The dried pellet was dissolved in 1 ml TE and stored at 4°C.

(b) Method 2 (alkaline lysis)

Chromosomal DNA extraction was carried out using the technique described by Cardy (1989) with the following modifications. Cells were harvested from a 500 ml overnight culture grown on Penassay by centrifugation in a Beckman 6x250 rotor (22,100g, 10 min, 4°C). The pellet was resuspended in 6 ml TE to which 3.75 ml of 0.25 M EDTA pH 8.0 and 50 mg lysozyme were added and gently mixed. After incubation at 37°C for 15 min, proteinase K (125 μl of 20 mg ml^{-1} stock) was added, followed slowly by 3.25 ml 10% (w/v) SDS and further incubated at 37°C until lysis occurred. 4 ml of 5M sodium perchlorate were added to aid membrane separation and placed at 60°C for 15 min with occasional stirring. An equal volume of TE saturated phenol:chloroform:iso-amylalcohol (25:24:1) was added and mixed gently. To facilitate complete separation of aqueous and organic phases, centrifugation was performed in a Beckman 8x50 rotor (39,200g, 30 min, 4°C) and the upper aqueous layer transferred to a fresh Oakridge tube. An equal volume of chloroform was added, mixed gently and centrifuged as before to separate aqueous and organic phases. Extraction was repeated twice and after the final extraction, the aqueous phase was transferred to a polycarbonate 250 ml centrifuge pot. NaCl (5 M stock) was added to 0.1 M followed by 2 volumes of ice cold ethanol. DNA was gently removed with a heat sealed pasteur pipette and washed several times with 70% (v/v) ethanol. The washed DNA was dried under vacuum for 30-60 min and then slowly resuspended overnight in 10 ml TE. Ribonuclease (RNase),

(20 mg ml^{-1} stock, Sigma) was added to 100 μl ml^{-1} and the solution incubated at 37°C for 30 min. The RNase treated DNA solution was then made up to 30 ml with TE into which 30 g CsCl were dissolved. Finally 3 ml of ethidium bromide (10 mg ml^{-1} stock) were added and the resulting mixture was placed in a Vti50 self-seal tube and centrifuged in a Beckman L8-70 centrifuge (196,400g, 16 hours, 20°C). The resulting DNA band was extracted and the ethidium bromide removed as previously described. 2 volumes of SDW and 6 volumes of ethanol were added and the DNA precipitated at -20°C overnight. The precipitate was pelleted in a Beckman 8x50 rotor (39,200g, 15 min, 4°C) and the pellet dried under vacuum. The dried DNA was resuspended in 1 ml TE and stored at 4°C.

2.13 Isolation of plasmid DNA

2.13.1 Large scale

(a) Method 1

A 250 ml overnight culture in Penassay was harvested by centrifugation in a Beckman 6x250 rotor (22,100g, 10 min, 4°C). The pellet was resuspended in 16.5 ml Tris-sucrose pH 8.0, 5 ml of lysozyme (5 mg ml^{-1} in 0.25 M Tris-HCl pH 8.0) was added and left on ice for 5 min. After addition of 0.25 M EDTA pH 8.0 (4.5 ml) and a further 5 min period on ice, 18 ml Lysis Mix were added and the mixture incubated at 42°C with occasional inversion until cleared. The cell debris was then removed as described previously (section 2.12(a)). 30 g of CsCl were added to 30 ml of supernatant and this was dissolved before adding 1 ml (10 mg ml^{-1}) ethidium bromide and loading in a Vti50 self-seal tube. The DNA was then purified by CsCl gradient centrifugation and the ethidium bromide removed, as previously described (section 2.12(a)). To the 5 ml DNA solution was added 1% (w/v) Sarkosyl (1.2 ml), 3 M Na Acetate pH 6.3 (1.2 ml), TE (4.6 ml) and 2 volumes of 96% (v/v) ethanol. The DNA was precipitated at -20°C

overnight or at -70°C for 3 hours. The DNA was then pelleted, dried under vacuum and resuspended in 500 μl of TE.

(b) Method 2

The alkaline lysis technique of Birnboim & Doly (1979) was used as described by Maniatis *et al.* (1982) except that the volumes were reduced by 20% to allow the use of Oakridge tubes. Additionally, solution II (alkaline SDS solution) was not placed on ice prior to use (as the SDS precipitates) and a centrifugation step (39,200g, 30 min, 15°C) was included prior to the CsCl gradient centrifugation to remove cell debris. After removal of ethidium bromide (section 2.12(a)), plasmid DNA was directly precipitated from CsCl as described previously (2.12(b)) and resuspended in 500 μl of TE.

2.13.2 Small scale (mini-prep)

A scaled down version of the large scale prep (2.13.1 (b)), working with 1.5 ml of culture, instead of 1 litre, was used. Solution II was again not placed on ice prior to use and also the 70% (v/v) ethanol wash of the DNA pellet was omitted as this resulted in loss of plasmid.

2.14 Restriction endonuclease digestion

Restriction endonucleases and restriction endonuclease buffers (x10) were obtained from Amersham or BRL, and used according to the manufacturer's instructions.

2.15 Agarose gel electrophoresis

To prepare horizontal slab gels, 1% (w/v) agarose, unless otherwise stated, was melted in TBE electrophoresis buffer and cooled slightly before pouring. DNA samples, restricted as appropriate, were mixed with $1/6^{th}$ volume of Loading Buffer Type IV and loaded into the gel slots. Electrophoresis was carried out with the gel completely submerged in buffer at 100-120 volts (V) or 60 V overnight. As DNA molecular weight (MW) markers, 10 μl of lambda$^+$ (bacteriophage DNA (50 ng ml^{-1}), digested with the restriction endonuclease *Hind*III, was used. This generated fragment sizes : 23.17, 9.46, 6.75, 4.26, 2.20, 1.92 and 0.58 kilobases (Kb). DNA was stained within the agarose gel with ethidium bromide (0.5 μl ml^{-1}) as described by Maniatis *et al.* (1982), and visualised by transillumination with short-wave UV light and photographed using Polaroid Type 665 black and white film.

2.16 Quantification of DNA

The concentration of DNA in a given sample was measured spectrophotometrically as described by Maniatis *et al.* (1982).

2.17 Oligo-labelling of DNA fragments by hexadeoxynucleotide primers

Purified DNA of recombinant plasmids, NTP705 and NTP707 (table 2.2), was prepared as described in section 2.13.1 (b) and the DNA probe fragments obtained by restricting the vector plasmids with the appropriate enzymes (Willshaw *et al.*, 1985, 1987). The digests were run on a 1% (w/v) low gelling temperature agarose (BRL) gel and the 0.75 Kb VT1 and 0.85 Kb VT2 fragments excised. To label the fragments to high specific activity, without prior purification from the gel slice, the procedure described by Feinberg & Vogelstein (1984) was followed with the following modifications. To 16.25 μl of DNA fragment was added in the following order :- 5 μl OLB buffer ; 1 μl BSA (10

mg ml^{-1}) ; 0.5 μl DNA Polymerase I (Amersham, Klenow-large fragment, cloned) and 2.5 μl ^{32}P-dC1P. The mixture was then incubated at room temperature for at least 5 hours or overnight. No stop buffer was added and the unincorporated nucleotides were removed using a spin column as follows :-

The end of a 1 ml syringe was plugged with a little siliconised glass wool and filled with Sephadex G50 (Pharmacia-equilibrated in TE pH 8.0 containing 0.1 M NaCl) through a long form pasteur pipette to within 5-10 mm of the top. A small bore needle, inserted into a 1.5 ml Eppendorf was added and the column placed, through a hole in the lid, into a plastic universal. The columns were centrifuged at 470g for 30 seconds (sec) in a MSE Mistral 1000 universal centrifuge and a fresh Eppendorf was added. 75 μl TE was added to the probe reaction and the mixture heated at 65^{o}C for 2 min to melt any agarose. This was then added to the column and centrifuged at 470g for 1 min. A further 100 μl TE was added and the column centrifuged as before. The Eppendorf was tested with a Geiger counter and if the probe had not been removed a further 100 μl TE was added and the column recentrifuged. 0.1 ml (10 mg ml^{-1}) sheared, heat treated Salmon Sperm DNA was added to the labelled probe, the mixture boiled for 10 min and kept on ice for 5 min before use.

2.18 Southern transfer of DNA

This method followed the basic protocol of Southern (1975), as adapted by Maniatis *et al.* (1982). Denaturation and neutralisation of DNA in the gel matrix were both carried out for 30 min at room temperature. The transfer buffer was 20 x SSC instead of 10 x SSC. Before placing the nitrocellulose on it, the neutralised gel was wetted with 2 x SSC to remove excess salt. The transfer of DNA was allowed to proceed overnight. When the filter was removed from the gel, it was soaked in 2 x SSC for a few min before being dried and baked, under vacuum at 80^{o}C for 2 hours.

2.19 Hybridisation of Southern filters

The procedure described by Maniatis *et al.* (1982) was essentially followed with the following modifications. Nitrocellulose filters were placed in heat-sealed Sterilin bags containing 40 ml pre-hybridisation solution (5 x SSPE, 5 x Denhardts, 0.1% (w/v) SDS), laid flat in a plastic container and incubated in a 65^{o}C water bath for 4-8 hours with shaking at 100 rpm. The pre-hybridisation solution was then replaced with 20 ml of fresh solution supplemented with the ^{32}P-labelled probe. Hybridisation was carried out for a minimum of 18 hours at 65^{o}C, 100 rpm followed by several stringent washes of 0.1 x SSC, 0.1% (w/v) SDS (250 ml) for 30 min at 65^{o}C, 100 rpm (unless otherwise stated). Filters were allowed to dry at room temperature, covered in cling-film and examined by autoradiography at -70^{o}C.

2.20 Autoradiography

Autoradiography was carried out at -70^{o}C for ^{32}P-labelled material and at room temperature for ^{35}S-labelled material using Harmer film cassettes (with intensifying screens for ^{32}P) and Fuji RX X-Ray film. Autoradiograms were developed in Kodak LX-24 developer and fixed in Kodak FX-40 according to the manufacturer's instructions.

2.21 Determination of toxin production

2.21.1 Polymyxin B release of Verotoxin. (Karmali *et al.*, 1985b)

0.5 ml of an overnight culture was used to inoculate 100 ml of Penassay which was then incubated at 37^{o}C with shaking for 5 hours. 30 ml of culture, transferred to an Oakridge tube was pelleted in a Beckman 8x50 rotor (16,000g, 10 min, 4^{o}C) and the supernatant discarded. The pellet was washed twice in sterile PBS, resuspended in 1 ml

Polymyxin B Sulphate (Sigma, 1 mg ml^{-1} in PBS) and incubated for 30 min at 37°C. After centrifugation in a MSE MicroCentaur (11,600g, 10 min) the supernatant was filtered through a 0.22 μm filter (Sartorius) and stored at 4°C.

2.21.2 Use of the Ricin Assay

Yeast ribosomes isolated from a culture of ABYS 1, and the recombinant ricin A chain used in these experiments were a gift from Dr. Martin Hartley, Department of Plant Biochemistry, Warwick University. These were stored at -70°C and 4°C respectively.

2.21.2.1 Treatment of RNA with ribosome inhibiting protein (RIP)

It was found necessary to pretreat Polymyxin B released VT samples (section 2.21.1) with β-mercaptoethanol (BME) to ensure formation of active A fragments. Typically, 10 μl of crude extract was mixed with 1 μl of BME (100 mM) and incubated for 1 hour at 30°C. Following this, 2 μl was mixed with 2 μl yeast ribosomes, 9 μl 2x ENDO buffer, 4 μl SDW, 2 μl 100 mM BME and 1 μl of a 1:5 dilution in DW of Vanadyl Ribonucleoside Complex (VRC), (BRL). This solution was incubated at 30°C for 20 min. As a positive control 2 μl of yeast ribosomes were incubated at 30°C for 20 min in the presence of 2 μl of recombinant ricin A chain, 8 μl SDW and 8 μl 2x ENDO buffer. To precipitate the RNA, 0.1 volume of 10% (w/v) SDS was added and the mixture extracted with an equal volume of phenol:chloroform (1:1). The aqueous layer was retained and the RNA precipitated overnight at -20°C after addition of 0.1 volume 2 M Na Acetate pH 6.0 plus 2.5 volumes ethanol. The precipitate was collected by centrifugation in a MSE MicroCentaur (11,600g, 20 min, 4°C,), the pellet washed in 70% (v/v) ethanol, repelleted as before and vacuum dried for approximately 15 min. The pellet was then dissolved in 10 μl SDW.

2.21.2.2 Aniline cleavage of depurinated RNA

The cleavage of ricin A chain-depurinated rRNA was first described by Endo *et al.* (1987). The method used here was essentially as described by May *et al.* (1989) with some modifications. 1 μl of RNA, treated as described in section 2.21.2.1 was incubated at 60^{o}C for 2 min with 20 μl 1 M aniline/acetic acid (pH 4.5), 0.1 volume 7 M NH_4 Acetate and 2.5 volumes of ethanol were added and the RNA precipitated on dry ice in the dark for 30 min. The RNA was then pelleted in a MSE MicroCentaur (11,600g, 4^{o}C, 15 min), the pellet washed in 70% (v/v) ethanol, dried under vacuum and dissolved in 20 μl of 60% (v/v) de-ionised formamide in 0.1 x Tris-phosphate (TPE) buffer. As a control an equivalent amount of non-aniline treated RNA sample was added to 20 μl 60% (v/v) formamide/TPE mixture. Samples were incubated for 5 min at 65^{o}C to denature the RNA and then cooled on ice. 3 μl of 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue was added to the samples.

2.21.2.3 Electrophoresis of depurinated RNA

Samples (+ or - aniline treatment), prepared as described in section 2.21.2.2 were loaded onto a 1.2% (w/v) agarose, 0.1 x TPE buffer, 50% (v/v) formamide gel (100 ml on 15 x 12 cm plates). The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer. To visualise the RNA, the gel was soaked for 10-20 min in 2 μg ml^{-1} ethidium bromide, destained in DW for 10 min and viewed on a UV transilluminator.

2.21.3 Use of the Vero cell cytotoxicity assay. (Konowalchuk *et al.*, 1977)

Stocks of Vero (African Green Monkey Kidney) cells, provided by Dr Iftihar Urabi (Warwick University) were grown as monolayers at 37^{o}C (5 % (v/v) CO_2 atmosphere), in medium 199 supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum and the

antibiotics, penicillin and streptomycin, to avoid contamination. Toxin activity was assayed in 6 well plastic tissue culture dishes into which approximately 10^6 cells per well in growth medium (2 ml) had been seeded, 1-2 days before. Samples for investigation were prepared essentially as decribed in section 2.21.1. except that Polymyxin B release of VT was from a 10ml overnight culture and this was pelleted in an MSE Mistral 1000 universal centrifuge (2,940g, 5 min). 200 μl of sample was added to the monolayer and the morphological and cytotoxic effects were recorded over the next 48 hours.

2.22 Determination of protein concentration

The Bio-Rad Protein Assay System (Bio-Rad Labs Ltd.) was used essentially as described by Bradford (1976). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs. This assay was routinely used in the determination of soluble protein concentration as it was much easier to use than the Lowry method, requiring 1 reagent and 5 min to perform as compared to 3 reagents and 30-40 min typical for the Lowry assay. Samples were diluted in SDW as required and Bovine Serum Albumin (BSA) was used as a standard. For each assay protein concentrations were determined by reference to a plot of protein concentration against the absorbance at 595nm (A595nm), prepared using a range of dilutions, (1-25 μg ml^{-1}, Microassay Procedure; 20-140 μg ml^{-1}, Standard Assay Procedure), of a 1 mg ml^{-1} BSA stock solution.

2.23 Preparation of cell free extracts

Typically (unless otherwise stated), 100 ml culture was centrifuged in a Beckman 6 x 250 rotor (22,100g, 10 min, 4^{o}C), the cell pellet resuspended to a high cell density in 1 ml ice-cold 10 mM Tris-HCl pH 7.0 and transferred to a 1.5 ml Eppendorf tube. Cells were then disrupted by sonication (Amplitude 12 μm peak to peak, 3 x 30 sec bursts

with 30 sec cooling inbetween) and the debris and unbroken cells removed by centrifugation in a MSE MicroCentaur (11,600g, 15 min, 4°C). The supernatant was filtered through a 0.22 μm membrane filter (Sartorius) frozen on dry ice and stored at -20°C.

2.24 Preparation of culture supernatants

2.24.1 Ammonium sulphate precipitation

Culture supernatant was obtained by centrifugation of a 100 ml culture in a 6 x 250 Beckman rotor (22,100g, 10 min, 4°C). NH_4SO_4 was added to 40% (w/v) and after stirring at 4°C for 20-30 min the mixture was pelleted (12,100g, 20 min, 4°C). NH_4SO_4 was added to the supernatant to 60% (w/v) and after further stirring at 4°C for 20-30 min, the mixture was pelleted as before. The pellet was resuspended in a small volume (2 ml) of Tris-HCl pH 7.0 and dialysed overnight with stirring at 4°C against 2 litres Tris-HCl pH 7.0 with 3 changes. The dialysed sample was then filter sterilised and stored as described in 2.23.

2.24.2 Concentration with an Amicon concentrator

Culture supernatant obtained as described in section 2.24.1. was dialysed overnight, with stirring at 4°C, against 2 litre volumes of Tris/glycine running buffer with 3 changes. 30 ml of dialysed supernatant was then concentrated to a small volume in an Amicon Micro-Ultrafiltration System (Model 8MC) using a YM-10 membrane (Amicon Corp.) to ensure that only macrosolute remained in the ultrafiltration chamber as concentrate. The concentrate was filter sterilised and stored as described in section 2.23.

2.25 Polyacrylamide gel electrophoresis (PAGE)

2.25.1 Slab gels

Proteins were analysed on 11% (w/v) denaturing, SDS polyacrylamide gels using a 4.5% (w/v) stacking gel (Laemmli, 1970). The constitution of buffer and acrylamide solutions for SDS-PAGE are shown in table 2.4.1. Table 2.4.2 gives the composition of the SDS polyacrylamide resolving and stacking gels. Samples for SDS-PAGE were mixed with an equal volume of sample buffer (table 2.4.1), boiled for 4 min, cooled and loaded onto the gel with a Hamilton syringe. Ammonium persulphate (AMPS) was always freshly prepared and the gel, once poured, was overlaid with water saturated butan-1-ol. Electrophoresis was carried out using a LKB 2001 Vertical gel system at a constant current of 40 mA, for approximately 3 hours or 8 mA overnight.

Table 2.4.1 Stock solutions for SDS-PAGE

Stock Solutions	Composition
Resolving Gel Buffer	0.75 M Tris-HCl pH 8.8 0.2% (w/v) SDS
Stacking Gel Buffer	0.25 M Tris-HCl pH6.8 0.2% (w/v) SDS
Acrylamide Stock	44% (w/v) Acrylamide 0.8% (w/v) Bisacrylamide
Ammonium Persulphate (AMPS)	1% (w/v)
TEMED	As supplied by manufacturer
Electrophoresis Buffer	0.025 M Tris 0.129 M Glycine 0.1% (w/v) SDS
Sample Buffer (x2)	0.025 M Tris-HCl pH 6.8 0.8% (w/v) SDS 10% (v/v) Glycerol 5% (v/v) β-mercaptoethanol Bromophenol Blue

Table 2.4.2 Composition of SDS-PAGE resolving & stacking gels

Stock Solution	Resolving Gel 11%	Stacking Gel 4.5%
Resolving Gel Buffer	27.00 ml	-
Stacking Gel Buffer	-	10.00 ml
Acrylamide Stock	13.60 ml	3.00 ml
Double Distilled Water	12.00 ml	7.00 ml
AMPS	1.90 ml	0.50 ml
TEMED	0.13 ml	0.04 ml

2.25.2 Silver staining

The method of Wray *et al.* (1981) was used as it is both relatively simple and rapid. Gels were soaked in 50% (v/v) methanol for a minimum of 8 hours with 3 changes. 1.6 g of silver nitrate was dissolved in 8 ml DW and slowly added to a solution containing 42 ml 0.36% (w/v) NaOH and 2.5 ml NH_3. The volume was made up to 200 ml with DW and this was then used to stain the gel for 15 min. After 2 washes in DW for 5 min each, the gel was soaked in developer (2.5 ml 1% (w/v) citric acid, 0.4 ml formaldehyde, made up to 500 ml with DW) until the bands appeared. The reaction was stopped with a solution containing 10% (v/v) acetic acid and 45% (v/v) methanol.

2.25.3 Destaining

Polyacrylamide gels were destained when necessary by soaking overnight in a solution containing 10% (v/v) Kodafix and 30% (v/v) methanol. The gels could then be restained as described in section 2.25.2.

2.26 Photography

Stained gels and autorads were routinely photographed from above using a Pentax SP500 camera with Kodak Panatomic X film (ASA 32).

2.27 Determination of ^{35}S-methionine incorporation into cell cultures

The method used was essentially as described by Swoboda *et al.* (1982) with the following modifications. To a 1.5 hour culture of *E. coli* O157:H7, grown on a 100 ml Penassay, was added ^{35}S-methionine (1300 Ci mM^{-1}) to a final concentration of 1 μCi ml^{-1} or 2 μCi ml^{-1}. Duplicate 0.5 ml samples were immediately removed and every 5 min thereafter, unless otherwise stated. Unlabelled L-methionine (100 mM stock) was added to 1 mM to prevent further incorporation of radiolabel, 2 ml ice-cold 5% (w/v) trichloroacetic acid (TCA) was added and the samples were left on ice for a minimum of 30 min to precipitate the cells. Samples were dried onto GF/C glass microfibre filters (Whatman), washed twice with ice-cold 5% (w/v) TCA, once with ice-cold ethanol and finally with a mixture of ethanol and ether (1:1). Filters were dried and counted in 5 ml Beckman EP Scintillation Cocktail in a LKB Minibeta Scintillation Counter. Efficiency of counting was 95% for ^{35}S methionine.

2.28 Pulse labelling of cultures with ^{35}S-methionine

7.7 μl of 10 μM L-methionine was added to growing cultures of *E. coli* O157:H7, one hour prior to the addition of radiolabel, to prime the cells for methionine uptake. At known time intervals, ^{35}S-methionine (1300 Ci ml^{-1}) was then added to a final concentration of 3 μCi ml^{-1}. After 10 min, incorporation was stopped by addition of unlabelled L-methionine to a final concentration of 1 mM and the cultures centrifuged to pellet the cells. Cell free extracts and culture supernatants were prepared and assayed for protein as peviously described (sections 2.22, 2.23 and 2.24.2). 15 μl of each

sample was filtered through GF/C glass microfibre filters and scintillation counted as previously described (section 2.27). Samples, adjusted to contain equal amounts of protein or counts of radiolabel, were electrophoresed on 11% (w/v) SDS polyacrylamide gels, silver stained, photographed and destained as described in sections 2.25 and 2.26. To increase the sensitivity of X-Ray film to ^{35}S labelled proteins, gels were impregnated with fluor (Amplify-Amersham) according to the manufacturer's instructions and dried under vacuum at 60^{o}C for 2 hours.

2.29 Protein markers

Protein MW markers of size range 14.4-94.0 KDa (Pharmacia) were reconstituted according to the manufacturer's instructions. Bromophenol blue was added and 5 μl routinely run on SDS-PAGE. ^{14}C Methylated proteins of size range 14.3-200 KDa (Amersham) were used as protein markers in pulse labelling experiments (2.28). Labelled proteins were mixed with an equal volume of sample buffer (x2), (table 2.4.1) and 10 μl run on SDS polyacrylamide gels.

2.30 Extraction of RNA

All glassware used in the preparation of RNA was baked overnight at 180^{o}C and all solutions were made using SDW previously treated with 0.1% (v/v) diethylpyrocarbonate (DEPC, Sigma). Also, all tubes, tips and reagents (except phenol and ethanol) were autoclaved before use and after baking, and/or autoclaving, all equipment and reagents were handled with gloves.

RNA extraction was carried out using the technique described by Crickmore (1987), with some modifications. Typically, unless otherwise stated, the relevant strain was grown overnight in 10 ml Penassay plus antibiotics, where appropriate. 1.5 ml was removed to an Eppendorf tube and the cells pelleted in a MSE MicroCentaur (11,600g,

3 min, 4°C). The supernatant was removed and the cells were resuspended in 450 μl of SAE (section 2.6). BRL ultra pure phenol (450 μl, equilibrated with 20 mM Na Acetate, pH 5.6) was added and an emulsion formed by vortexing. The mixture was then incubated at 65°C with regular mixing. After centrifugation (11,600g, 10 min), the phenol extraction was repeated on the aqueous phase, after which the RNA in the aqueous phase was precipitated at -70°C for 30 min by the addition of 1 ml of ethanol. The RNA was then pelleted (11,600g, 10 min), resuspended in 333 μl of SAE and precipitated as before. After pelleting for the second time the RNA was dried and resuspended in 200 μl of SAE, before being stored at -70°C.

2.31 Quantification of RNA

The concentration of RNA in a given sample was measured spectrophotometrically as described by Maniatis *et al.* (1982).

2.32 Electrophoresis of RNA

The RNA to be probed was electrophoresed on a formaldehyde denaturing gel. The gel (1.5% (w/v) agarose) was prepared by boiling 2.25 g of agarose in 93 ml DEPC treated water. When the agarose had dissolved, 30 ml of 5x MOPS was added followed by 27 ml of formaldehyde and the gel was then immediately poured. Typically, unless otherwise stated, 10 μg of RNA in a known volume was mixed with 3 volumes of formaldehyde loading buffer (FLB) and denatured by heating at 68°C for 3 min before loading. 5 μl of RNA MW markers (Gibco, BRL) of size range 0.24-9.49 Kb and 8 μl each of unlabelled VT1 and VT2 DNA probe fragments were also denatured in FLB and loaded onto the gel. The gel was run at 80 V during the day or at 30 V overnight in 1x MOPS buffer.

2.33 Northern blotting

After electrophoresis the gel was washed in water, photographed, then soaked twice in 10x SSC for 20 min. The RNA was then transferred to nitrocellulose exactly as for Southern Blotting (section 2.18) and probed as follows:-

(a) Method 1

Northern filters were routinely probed exactly as for Southern filters (section 2.19). However, filters were washed gradually with increasing stringency, typically being, (unless otherwise stated) :-

(1) 2 washes for 20-30 min at room temperature (2x SSC, 0.1% (w/v) SDS).

(2) 2 washes for 20-30 min at room temperature (1x SSC, 0.1% (w/v) SDS).

(3) 2 washes for 20-30 min at room temperature (0.1x SSC, 0.1% (w/v) SDS).

(b) Method 2

Nitrocellulose filters were probed as described in section 2.19 for Southern filters with the following modifications. Filters were incubated in pre-hybridisation solution (5x SSC, 50% (v/v) formamide, 1x Denhardts) at 42^{o}C. Hybridisation was carried out at 42^{o}C and washes consisted of :-

(1) 2 washes for 20-30 min at room temperature (2x SSPE,0.1% (w/v) SDS).

(2) 2 washes for 20-30 min at room temperature (0.1x SSPE, 0.1% (w/v) SDS).

(3) 2 washes for 20-30 min at 50^{o}C (0.1x SSPE, 0.1% (w/v) SDS).

2.34 Treatment of RNA with DNase

DNase buffer was made by dissolving 0.203 g $MgCl_2$ (100 mM) in 10 ml of 100 mM Na Acetate pH 5.6. To 50 μl of RNA, extracted as described in section 2.30 was added 350

μl DNase buffer and 1 μl of DNase-RNase free (BRL). This was incubated at 37^{o}C for 30 min then phenol extracted and ethanol precipitated for 1 hour at -70^{o}C. The dried pellet was dissolved in 40 μl of SAE.

2.35 RNA half-life determination

50 ml Penassay was inoculated with 250 μl of an overnight culture of the relevant strain and incubated at 37^{o}C with shaking until the late exponential phase was reached (OD600nm of approximately 1.5). Rifampicin (Sigma, 30 mg ml^{-1} in methanol) was added to a final concentration of 100 μg ml^{-1}, the time noted and 1 ml immediately removed to ice and pelleted in a MSE MicroCentaur (11,600g, 3 min, 4^{o}C). The supernatant was removed and the pellet frozen on dry ice. Further 1 ml samples were removed after 5, 10, 20, 30, 40, 50, 60 min incubation with rifampicin and the cell pellets collected as described above. When all the cell pellets had been obtained, RNA was extracted from them as described in section 2.31, except that the RNA was finally resuspended in 100 μl of SAE before being stored at -70^{o}C. Samples, adjusted to contain 10 μg of RNA, were denatured in FLB, divided into two and loaded onto two formaldehyde gels so that they were identical. Gels were run overnight, Northern blotted and subsequently probed with either/or ^{32}P-dCTP labelled VT1 and VT2 probe fragments as described in sections 2.32 and 2.33(a).

2.36 Preparation of RNA from the growth cycle

200 ml Penassay plus appropriate antibiotics was inoculated with 1.0 ml of an overnight culture of *E. coli* 6OR746 or 6OR363. At known time intervals, 3 ml of culture was pelleted and RNA extracted as described in section 2.30. Samples were resuspended in increasing amounts of SAE (50-200 μl) and the concentration of RNA in each sample determined (section 2.31). Samples adjusted to contain 15 μg RNA were denatured in

FLB, loaded onto a formaldehyde gel in duplicate and Northern blotted as previously described (sections 2.32 and 2.33(a)).

2.37 Bacterial transformation

Two methods were employed to introduce DNA into bacterial cells:- Electroporation and the induction of "Pseudocompetence" by pretreatment with Ca^{2+}.

2.37.1 Electroporation

Electroporation, one of the most recent advances for the introduction of DNA into cells, involves the application of a brief, high voltage pulse to a suspension of cells and DNA, resulting in a transient membrane permeability and the subsequent uptake of DNA.

Cells for electroporation were propagated in 100 ml LB and harvested at an OD600nm of 0.6-0.8. Cells were pelleted in a Beckman 6x250 rotor (22,100g, 10 min, $4^{o}C$), the supernatant discarded and the cell pellet washed twice in 50 ml ice-cold SDW. The pellet was then washed in 25 ml ice-cold sterile 10% (v/v) glycerol, repelleted and resuspended in 200 μl of the latter. The cells were frozen on dry ice and stored at -$70^{o}C$.

Electroporation was carried out using a Gene Pulser^{TM} (Bio-Rad Labs USA), set to 2.5 KV, a capacitance of 25 μF and a resistance of 200 ohms. 1 μl of CsCl density gradient purified plasmid DNA was mixed with 40 μl of electroporatable cells and left on ice for 1 min before being transferred to an ice-cold 0.2 cm electroporation cuvette. The cell/DNA mixture was spread evenly across the bottom between the chilled electrodes, the cuvette placed in the safety chamber and the pulse applied. Following the pulse, 1 ml of LB was added and the mixture transferred to an Eppendorf tube and

incubated at 37°C for 1 hour to allow for expression of the antibiotic resistance genes. 100 μl aliquots were then plated onto selective agar to screen for transformants.

2.37.2 The calcium chloride procedure

The technique used was essentially that described by Holland (1983). 0.5 ml of overnight culture was used to inoculate 50 ml of LB in a 100 ml flask. This culture was grown to an OD600nm of approximately 0.5 and the cells were then held on ice for 10 min. From this point, all operations were carried out on ice and using ice-cold solutions. The cells were pelleted by minimal centrifugation in a multex angled centrifuge, the supernatant carefully removed and the pellet resuspended in 0.5 volume of $CaCl_2$ (100 mM). After a further 10 min on ice, the culture was repelleted, resuspended in 0.05 the original volume (2.5 ml) of 100 mM $CaCl_2$ and was held on ice for a minimum of 1 hour (Dagert & Ehrlich, 1979).

200 μl of competent cells were aliquoted into a 1.5 ml Eppendorf tube and 1 μl DNA added to the cells, mixed gently and then left on ice for 30 min. This was then heat shocked at 42°C for 2 min and returned to ice for 15 min. Two volumes of LB were added, incubated for 1 hour at 37°C, to allow expression of antibiotic resistance genes after which 100 μl aliquots were plated onto selective media.

2.38 Transduction with lambda Tn*phoA*

Lambda Tn*phoA* (table 2.3) was provided by Dr. G. Salmond, Dept. of Biological Sciences, Warwick University. A single transformant of *E. coli* CC118 (pNTP707) was grown overnight in LB/Tc and 100 μl used to inoculate 10 ml fresh LB/Tc. This was grown to exponential phase at 37°C with shaking (250 rpm). The culture was pelleted in a MSE Mistral 1000 universal centrifuge (2,940g, 5 min, room temperature), the pellet resuspended in 1 ml of LB/Mal/Mg^{2+}/Tc and 100 μl of lambda Tn*phoA* was

added. After static incubation at 37°C for 30 min to allow for phage adsorption and infection, 10 ml LB/Tc was added and the culture incubated for 1 hour at 37°C (250 rpm) to allow for expression of Kn^r. The culture was pelleted as before, resuspended in 1 ml LB/Tc and 100 μl aliquots spread on LB plates containing Tc, Kn (300 $\mu g\ ml^{-1}$) and XP (Sigma, 40 $\mu g\ ml^{-1}$ in DMSO) and incubated at 37°C for two days. Mini plasmid preparations (2.13.2) of blue colonies were used to transform freshly competent *E.coli* CC118 as described in section 2.37.2. Individual blue colonies on LB plates containing Tc, Kn (30 $\mu g\ ml^{-1}$) and XP after 2 days at 37°C, contained in frame fusions of Tn*phoA* to secreted gene products on plasmid NTP707.

2.39 DNA Sequencing

To determine the site of insertion of Tn*phoA* within pSLF22 and pSLF34 two methods were employed :-

(1) Dideoxynucleotide chain termination method of DNA sequencing (Sanger *et al.*, 1977; Sanger *et al.*, 1980) - section 2.39.2.

(2) Direct plasmid sequencing - section 2.39.3.

2.39.1 Preparation of fragments from agarose gel

(a) Method 1

The excised fragment was placed in dialysis tubing (prepared as described by Maniatis *et al.*, 198?) with a small volume of 0.5 x TBE (approximately 200 μl). All air bubbles were removed and a current of 40 mA was applied across the dialysis tubing in a minigel tank buffered with 1 x TBE for 20 min. The polarity was then reversed for 30 sec to remove any DNA from the side of the dialysis tubing and the DNA in solution removed to an Eppendorf tube. The solution was extracted twice with phenol, once with TE saturated chloroform and the DNA precipitated at -20°C for a minimum of 3 hours

upon addition of 0.1 volumes 3 M Na Acetate and 2 volumes ethanol. DNA was recovered by centrifugation in a MSE MicroCentaur (11,600g, 10 min, 4°C), washed with 70% (v/v) ethanol, repelleted and dried under vacuum for 5 min. The dried pellet was resuspended in 20 μl of TE.

(b) Method 2 (Gene Clean)

The excised fragment was weighed and the DNA purified from the gel slice using the Gene Clean II Kit (Bio 101) as described by the manufacturer. The DNA fragment was contained within a final volume of 10 μl TE.

2.39.2 Dideoxynucleotide chain termination method of DNA sequencing

2.39.2.1 Ligation of DNA

1 μl M13mp19 RF DNA (Gibco BRL) was digested with the appropriate restriction endonucleases, gene cleaned and resuspended in 10 μl TE. 5 μl of purified DNA fragment containing the fusion joint was ligated with 1 μl M13mp19 digest using T4 DNA ligase (Amersham) according to the recommendation of the supplier, at 15°C for a minimum of 18 hours.

2.39.2.2 Isolation of M13 recombinants

Competent *E.coli* TG1 cells were transformed with between 1-3 μl of ligation mix using the method described for plasmid transformation (section 2.37.2), except that following heat shock, cells were added to 3 ml, 0.7% (w/v) TYE soft agar to which had been added 60 μl of 2% (w/v) X-gal (20 mg ml^{-1} in DMF) and 30 μl of 2.5% (w/v) IPTG (25 mg ml^{-1}). This was immediately poured onto fresh TYE plates and allowed to set. After overnight incubation at 37°C, lawns were examined for plaque formation. Clear

plaques were indicative of TG1 infection with recombinant M13 vectors, whereas transfection with non-recombinant M13 vectors generated blue plaques.

2.39.2.3 Template preparation

Template preparation was essentially as described by Bankier *et al.* (1986), except that *E. coli* TG1 was used as the host for M13 recombinant propagation. After propagation of phage for 5 hours, cultures were transferred to 1.5 ml Eppendorf tubes and centrifuged in an MSE MicroCentaur (11,600g, 20 min), to precipitate the bacteria. Supernatants were removed to a fresh Eppendorf tube, 200 μl of PEG (6,000 MW, 20% (w/v)) was added and the mixture briefly vortexed and left standing at room temperature for 10-15 min. The mixture was centrifuged for a further 20 min and the supernatant removed. Any residual supernatant was removed after a brief 30 sec spin. The resulting phage pellet was resuspended in 100 μl TE, allowed to stand at room temperature for 10 min and 50 μl of TE saturated phenol was then added, vortexed well and centrifuged for 5 min at room temperature. To 80 μl of the aqueous phase, placed in a fresh Eppendorf was added, 40 μl of 7.5 M NH_4Ac and 200 μl ethanol. This was vortexed and placed at -20^{o}C overnight. After centrifugation, the pellet was dried under vacuum, resuspended in 20 μl TE and stored at -20^{o}C.

2.39.2.4 Dideoxy sequencing

The extended method of DNA sequencing with Klenow fragment was used as described by Cardy (1989). The primer used corresponded to a 19 bp portion of the IS50L of Tn5 and was provided by Dr D. Hodgeson, Dept. of Biological Sciences, University of Warwick. For the template-primer annealing reaction, 2 μl of template DNA was mixed with 2 μl sequencing buffer (x5), 2 μl of Tn5 primer and SDW up to 10 μl final volume and incubated for 1 hour at 55^{o}C. All subsequent steps (sequencing reactions and denaturation) were carried out in microtitre trays. The products of the

primer extension reactions were denatured in an oven (Mini/696/Clad) at 80°C for 15 min and placed immediately on ice prior to loading the whole of each reaction mixture on a 6% (w/v) polyacrylamide, tris-borate-urea sequencing gel (section 2.39.4).

2.39.3 Direct plasmid sequencing

2.39.3.1 End labelling of primer

3 μl of Tn5 primer was mixed with 1 μl 0.1 M DTT, 2 μl kinase buffer (x10), 4 μl (80 μCi) ^{32}P-dATP, 2 μl T4 polynucleotide kinase and 8 μl SDW in a 0.5 ml Eppendorf tube. This was incubated at 37°C for 40 min after which the enzyme was inactivated by heating the mixture in a dry block at 68°C for 10 min. 40 μl of SDW was added followed by 240 μl of 5 M NH_4 Acetate and mixed well. 750 μl of ice-cold ethanol was then added, mixed well and left at -20°C for a minimum of 1 hour. The precipitate was pelleted in a MSE MicroCentaur (11,600g, 20 min, 4°C) and the supernatant totally removed. The pellet was dried under vacuum for 10 min and resuspended in 4 μl of SDW.

2.39.3.2 Denaturation of DNA

5-10 μg of plasmid DNA, in a total volume of 8 μl was mixed with 2 μl 5x denaturing solution (1 M NaOH, 1 mM EDTA pH 8.0) and incubated at 37°C for 30 min. Salts were then removed from the denatured DNA using a spin column as follows:-

The lid of a 0.5 ml Eppendorf tube was removed, the base pierced twice with a small bore needle and the bottom plugged with a small amount of glass wool. The Eppendorf was filled completely with Sepharose CL4B (Pharmacia) and placed in a 1.5 ml Eppendorf tube, the lid of which had also been removed. This was centrifuged a total of 4 times in a MSE MicroCentaur (11,600g, 30 sec) and after each centrifugation any

buffer present in the base of the 1.5 ml Eppendorf was discarded. The column was then washed with 10 μl of SDW in a further 30 sec centrifugation step. The prepared column was placed in a fresh 1.5 ml Eppendorf tube, the denatured DNA was added and the column centrifuged as before for approximately 40 sec. 6 μl of the denatured DNA solution which passed through the column was immediately removed to primer/reaction buffer mix (2 μl Sequenase reaction buffer and 2 μl endlabelled Tn5 primer - section 2.39.3.1) and incubated at 37^{o}C for 30 min.

2.39.3.3 Plasmid sequencing using Sequenase Version 2.0

The Sequenase Version 2.0 kit (US Biochemical Corp.) was used as described by the manufacturer, except that no labelling step was performed. To 10 μl of template-primer mix was added 1 μl DTT (0.1 M), 2.5 μl SDW and 2.0 μl dilute Sequenase enzyme (diluted 1:8 with ice-cold dilution buffer). This was immediately split into 4, 3.5 μl amounts in 4 Eppendorf tubes each containing a single prewarmed 2.5 μl volume of one of the dideoxynucleotide termination mixes G,A,T and C. The 4 mixes were placed at 37^{o}C for 3-5 min, 4 μl of stop solution was added, the mixtures placed in an 80^{o}C water bath for 2.5 min and then immediately removed to ice. Reaction mixes were loaded onto a 6% (w/v) polyacrylamide tris-borate-urea sequencing gel, electrophoresed and fixed as described in section 2.39.4.

2.39.4 Sequencing gel electrophoresis

A stock solution of 38% (w/v) acrylamide, 2% (w/v) N,N'-methylenebisacrylamide (Bisacrylamide) was used to prepare the working solution for the gels. Typically a 6% (w/v) polyacrylamide wedge gel was made by dissolving 46 g of urea in 15 ml 40% (w/v) acrylamide (19:1 Bis) stock, 10 ml 10x TBE and SDW up to 100 ml final volume. The acrylamide was polymerised by the addition of 667 μl freshly prepared 10% (w/v) AMPS plus 40 μl TEMED and immediately poured. Electrophoresis was carried out at 55 W in 1x TBE after which gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic

acid for 15 min, transferred to Whatman 3 MM paper and dried under vacuum at 80°C for a minimum of 2 hours.

2.39.5 Sequence analysis

Autoradiography was carried out at -70°C as described in section 2.20. All DNA sequence derived from this work was recorded and the site of insertion of Tn*phoA* determined by comparison with published sequence data for VT2 (Jackson *et al.*, 1987b).

2.40 Assessment of plasmid stability

To determine the level of plasmid stability in each strain, 50 μl aliquots of an overnight of each was used to inoculate 10 ml volumes of LB containing decreasing concentrations of antibiotics (table 2.5). Cultures were then incubated overnight at 37°C with shaking (250 rpm), after which serial dilutions in PBS were plated on LB and LB/Kn/Ap (pSC105) or LB/Kn/Tc (pSLF22) agar plates. These were incubated overnight and colony counts made to determine the level of plasmid retention.

Table 2.5 Plasmid stability - Antibiotic concentrations

Number	Kanamycin ($\mu g\ ml^{-1}$)	Ampicillin ($\mu g\ ml^{-1}$)	Tetracycline ($\mu g\ ml^{-1}$)
1	50.0	100.0	15.0
2	25.0	50.0	7.5
3	12.5	25.0	3.75
4	6.25	12.5	1.875
5	0.0	0.0	0.0

Strain E3787 (pSC105) antibiotic combination = Kn + Ap
Strain E32511 (pSLF22) antibiotic combination = Kn + Tc

2.41 Plasmid curing under phosphate limitation. (Roeder and Collmer, 1985)

Strains E3787 (pSC105) and E32511 (pSLF22) were grown overnight in 10 ml LB plus appropriate antibiotics at 37°C with shaking (250 rpm). 10 μl of each was used to inoculate 5 ml of low phosphate media (section 2.3.5) which was then incubated at 37°C with shaking (250 rpm) for 48 hours, selecting for maintenance of Kn^r. After this time 10 μl of each culture were used to inoculate a further 5 ml of low phosphate media containing Kn and incubated for a further 48 hours as before. After each 48 hour incubation, serial dilutions in PBS were plated onto LB/Kn and LB/Ap or LB/Tc as appropriate. 50 colonies of each culture displaying Kn^r were replica plated onto LB/Ap or LB/Tc plates to screen for Ap^s or Tc^s isolates. Additionally colonies displaying Ap^r or Tc^r were replica plated onto LB/Kn plates. All plates were incubated at 37°C overnight.

2.42 Preparation of periplasmic proteins

Periplasmic proteins were released from cultures in response to cold osmotic shock treatment according to the method of Neu and Heppel (1965). Typically, 5 ml of an overnight culture was buffered by addition of 0.5 ml Tris-HCl (0.5 M, pH 7.8) and incubated at room temperature for 10 min. The cells were then pelleted in a MSE Chilspin (5,280g, 5 min), the pellet resuspended in 800 μl sucrose solution and transferred to 1.5 ml Eppendorf tubes. After 10 min incubation at 30°C, cells were repelleted in a MSE MicroCentaur (11,600g, 1 min) and the supernatant carefully removed. Cells were rapidly resuspended in 0.5 ml ice-cold SDW and left on ice for 10 min. Shocked cells were then removed by centrifugation (11,600g, 5 min) and the resulting shock fluid used in Western blot analysis.

2.43 Western blotting

2.43.1 Transfer of proteins separated by PAGE to nitrocellulose

Periplasmic proteins (section 2.42) were assayed for protein as described in section 2.22 and samples, adjusted to contain equal amounts of protein were mixed with $1/5^{th}$ volume of Loading Buffer (x5, boiled for 4 min and electrophoresed on 11% (w/v) SDS polyacrylamide gels as described in section 2.25. 5 μl of 1 mg ml^{-1} bacterial alkaline phoshatase (*E.coli* Type IIIL, Sigma) was also loaded on the gels. After electrophoresis the stacking gel was removed, the gel soaked in Western Transfer Buffer (WTB) for 10-15 min and the proteins transferred to nitrocellulose as follows. A nitrocellulose filter was cut to the size of the polyacrylamide gel, soaked in WTB and placed on top of the gel with care being taken to remove air bubbles between the gel and the nitrocellulose. Using a BioRad Transblot tank and power supply according to the manufacturer's instructions, transfer of proteins from gel to nitrocellulose was carried out at 40 V for 3 hours. After transfer the nitrocellulose filter was rinsed in DW to remove excess salt and the filter stained to visualise protein bands by washing for 10 min in Ponceau S (0.5% (w/v) in 5% (v/v) TCA). The position of the slots and protein markers was noted and the stain was then removed by washing in 20 ml PBS for 10 min, followed by addition of 10 ml fresh PBS containing 2% (w/v) dried milk (Marvel). The filter was washed in the latter for a minimum of 1 hour to allow protein to bind non-specifically to the nitrocellulose. The PBS:2% (w/v) Marvel was replaced with a fresh 10 ml volume of PBS:2% (w/v) Marvel containing the primary antibody, rabbit antibacterial alkaline phosphatase (NBL), at a dilution of 1:500. The filter was allowed to shake in this solution overnight at room temperature and was then washed three times for 10 min each time in 10 ml PBS containing 0.1% (v/v) Tween 20.

2.43.2 Detection of antigen-antibody complexes using a horseradish peroxidase colour reaction

Peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody and 4-chloro-1-naphthol (Sigma) as the colour reaction agent. After the washes in PBS:0.1% (v/v) Tween 20 described in section 2.43.1 the filter was transferred to fresh PBS:0.1% (v/v) Tween 20 (10 ml) containing a 1:300 dilution of secondary antibody and was left shaking for 1-2 hours at room temperature. After two 10 min washes in 10 ml PBS:0.1% (v/v) Tween 20 and two washes in 10 ml PBS, the filter was transferred to a solution containing 1.5% (w/v) NaCl, 1% (v/v) 1M Tris-HCl (pH 7.5), 0.03% (w/v) 4-chloro-1-naphthol and 10% (v/v) methanol. 50 μl H_2O_2 (Fisons) was then added to initiate the staining reaction. Before the reaction reached completion the filter was washed with DW (3 changes in 30 min), air dried, photographed and stored in the dark.

2.44 Enzyme assays

2.44.1 Sample preparation

(a) Method 1 (sonication of cells)

5 ml of culture (of known OD600nm) was pelleted in a MSE Chilspin (5,280g, 5 min). A sample of the supernatant was transferred to a 1.5 ml Eppendorf and immediately frozen on dry ice and stored at -20^{o}C. The cell pellet was resuspended in 5 ml Tris-HCl, pH 8.0 and the OD600nm was taken. Cells were then disrupted by sonication in a 25 ml glass beaker (Amplitude 6 μm peak to peak, 3 x 30 seconds bursts with 30 seconds cooling in between) and the cell debris removed by centrifugation in a MSE Chilspin as before. The cell free extract was then frozen on dry ice and stored at -20^{o}C.

(b) Method 2 (permeabilisation of cells)

5 ml of culture (of known OD600nm) was pelleted in an MSE Chilspin (5,280g, 5 min). A sample of the supernatant was transferred to a 1.5 ml eppendorf, immediately frozen on dry ice and stored at -20°C. Cells were resuspended in 5 ml Tris-HCl pH 8.0 and 1 ml aliquots immediately frozen on dry ice and stored at -20°C. For enzyme assays cells were thawed and permeabilised essentially as described by Michaelis *et al.* (1983), except that 10 μl 10% (w/v) SDS and 20 μl chloroform were added and the mixture incubated at 37°C for 5 min.

2.44.2 Alkaline phosphatase

Alkaline phosphatase (PhoA) activity was assayed according to the method of Brickman and Beckwith (1975). A 0.1 ml aliquot of sample to be assayed was mixed with 0.9 ml Tris-HCl (pH 8.0) and the reaction started by addition of 0.1 ml freshly prepared Sigma 104 (4 mg ml^{-1} in 1 M Tris-HCl pH 8.0). The mixture was incubated at 37°C and the time monitored until a yellow colour appeared. The reaction was stopped by addition of 0.1 ml of 1 M KH_2PO_4 and the OD550nm and OD420nm were read against a water blank. PhoA activity was expressed in units per OD600nm of bacterial cells using the following formula.

$$\text{Enzyme Activity (U)} = 1000 \times \frac{OD420 - (1.75 \times OD550)}{\text{time} \times OD600nm} \times \text{Dilution Factor}$$

2.44.3 Beta-lactamase

β-lactamase activity was assayed according to the method of O'Callaghan *et al.* (1982) in 1 ml microcuvettes. Assay mixtures contained 20-100 μl of sample made up to 800 μl with 0.1 M potassium phosphate buffer (pH 7.0). 20 μl of Nitrocephin (4 mg ml^{-1} in

DMSO) was added and the A500nm at 30^{o}C followed using a PYE Unicam SP1800 spectrophotometer. Enzyme activity in milli-units (mU) was expressed as the rate of hydrolysis of nitrocephin per minute per ml sample, normalised to the OD600nm of bacterial cells using the following formula:

$$\text{Enzyme Activity (mU)} = 1000 \times \frac{(\text{A500/min})}{\text{OD600nm}} \times \text{Dilution factor}$$

2.45 Verotoxin expression during growth

E.coli strains CC118, CC118 (pSC105), E32511 and E32511 SLF22/1 were grown overnight in 10 ml of LB plus the appropriate antibiotics. Overnight cultures were then pelleted in a MSE Mistral 1000 (2,940g, 5 min), the supernatant discarded and the pellet resuspended in 10 ml of Tris-HCl pH 8.0. 1 ml of each overnight was inoculated into a 200 ml volume of LB containing the appropriate antibiotics. Cultures were then incubated at 37^{o}C with shaking (250 rpm). Every hour samples were taken to determine the OD600nm of each culture. Additionally, every 2 hours (unless otherwise stated) samples of cultures CC118 and CC118 (pSC105) were taken and prepared as described in section 2.44.1(a) and samples of cultures E32511 and E32511 SLF22/1 were taken and prepared as described in section 2.44.1(b). Samples of CC118 and CC118 (pSC105) were assayed for PhoA and β-lactamase activity (sections 2.44.2 and 2.44.3), whereas samples of E32511 and E32511 SLF22/1 were assayed for PhoA activity alone (section 2.44.2).

2.46 Verotoxin expression under iron limitation

E.coli strains CC118, CC118 (pSC105), E32511 and E32511 SLF22/1 were grown overnight in 10 ml of LB plus the appropriate antibiotics. Overnight cultures were then pelleted in a MSE Mistral 1000 (2,940g, 5 min), the supernatant discarded and the

pellet resuspended in 10 ml of Tris-HCl pH 8.0. Cultures were split into two, recentrifuged and the pellets resuspended in either 5 ml prewarmed (37^{o}C, 250 rpm, overnight) iron replete LB (section 2.3.2) or 5 ml prewarmed iron limited LB (section 2.3.3). Kanamycin had been added to the LB at the prewarming stage where required. 1 ml of each overnight culture resuspended in iron replete LB was inoculated into a 200 ml volume of prewarmed LB containing the appropriate antibiotics whereas, 1 ml of each overnight culture resuspended in iron limited LB was inoculated into a 200 ml volume of prewarmed iron limited LB containing the appropriate antibiotics. Cultures were then incubated at 37^{o}C with shaking (250 rpm). Every hour, samples were taken to determine the OD600nm of each culture. Additionally, every 2 hours (unless otherwise stated), samples of cultures were taken for enzyme assay as described in section 2.45.

2.47 Expression of VT1 under iron limitation in a glass universal

Overnight cultures of CC118 and CC118 (pSC105) were resuspended in either iron replete or iron limited LB as described in section 2.46. 50 μl of each overnight culture, resuspended in iron replete LB was inoculated into 6 x 5 ml volumes of iron replete LB in glass Universals, whereas 50 μl of each overnight culture resuspended in iron limited LB was inoculated into 6 x 5ml volumes of iron limited LB in glass universals. Antibiotics were included where appropriate. Cultures were incubated at 37^{o}C with shaking (250 rpm). After 8 and 24 hours incubation, triplicate 5 ml cultures of each strain grown under iron replete and iron limited conditions were pelleted in a MSE Mistral 1000 and prepared for enzyme assay as described in section 2.44.1(a). Samples were assayed for PhoA and β-lactamase activity as described in section 2.44.2 and 2.44.3.

2.48 VT expression under anaerobic conditions

500 ml Quickfit subaseal flasks were specially made with a pyrex test-tube side arm. For growth under anaerobic conditions, 2 ml of saturated pyrogallol was added to the side arm of a flask containing 100 ml of LB and the flask and side arm air spaces gased with sterile nitrogen gas for 5 min. 2.5 ml of a solution containing 10% (w/v) NaOH and 15% (w/v) K_2CO_3 was then added to the side arm to ensure total anaerobiosis. Flasks containing the appropriate antibiotics were inoculated with 0.5 ml of an overnight culture and incubated at 37^{o}C with shaking (250 rpm). For comparison purposes, strains were cultured under aerobic conditions (no nitrogen purging or pyrogallol) in parallel. Every hour, samples were taken to determine the OD600nm of each culture. Additionally, every 2 hours (unless otherwise stated) samples were taken for enzyme assay as described in section 2.45.

CHAPTER THREE

3. Characterisation of Verotoxin producing strains

3.1 Introduction

The aim of this project was to examine the regulation of verotoxin (VT) production in laboratory culture. The strains used in this study and their source have been presented in table 2.1.

Strains C600(933J) and C600(933W) are *E. coli* C600 strains lysogenised with the VT1 encoding phage 933J of *E. coli* O157:H7 strain 933 and the VT2 encoding phage 933W of strain 933 respectively. *E. coli* strains E3787/H19 (O26:H11) and E32511 (O157:H$^-$) were isolated from cases of diarrhoea and HUS respectively. Production of VT1 by strain E3787 and VT2 by strain E32511 has been reported previously (Willshaw *et al.*, 1987). The *E. coli* strain termed Clinical Isolate (CI) was of unknown serotype and was isolated from a case of bloody diarrhoea at Warwick Hospital.

The disease association of *E. coli* strains O157:H7 (ATCC 35150) and O26:H11 (NCTC 8781) is unknown but both serotypes are commonly associated with haemorrhagic diarrhoeal disease and have been classified as enterohaemorrhagic *E. coli* (EHEC), as opposed to VTEC by Levine (1987), (section 1.5.4).

3.1.1 Aims

As a prerequisite for the development of an assay system to specifically quantify VT1 and VT2 gene expression, the strains described above (unless otherwise stated), were subjected to routine studies to define growth characteristics and nutritional requirements. Additional studies to define/confirm toxin genotypes, presence of phage and toxin production were also performed. The results from these studies are described in this chapter.

3.2 Growth characteristics of VTEC

Growth of all the strains described in section 3.1.1, including *E. coli* K12, was followed by recording the optical density at 600nm (OD600nm) at known time intervals (section 2.7.3). The viable count (section 2.7.4), total cell count and cell volume were also determined (section 2.7.5).

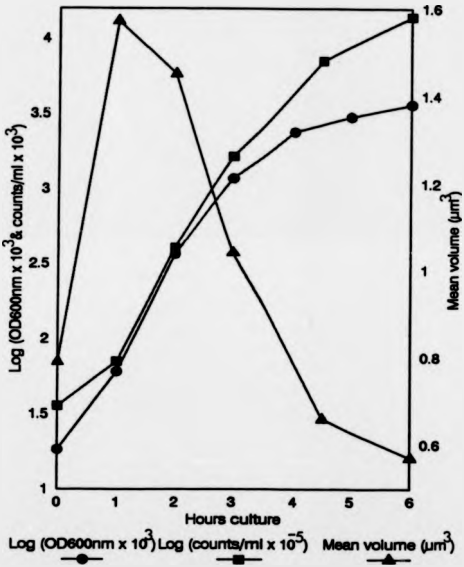
All strains displayed very similar growth characteristics and therefore only the data obtained for *E. coli* O157:H7 (ATCC 35150) is shown (figure 3.1). Under the conditions employed, growth was characteristically exponential (log phase) with a growth rate, determined as described by Prescott *et al.* (1990), of 0.98 generations per hour. After approximately 4 hours incubation, the culture entered stationary phase and the cells became smaller, as a result of dividing faster than they grew. Throughout the time course of the experiment there was no drop in viability of the culture (results not shown).

Konowalchuk *et al.* (1978) originally found Evans medium (Evans *et al.*, 1973), to be optimal for VT production. However, Penassay broth was chosen for routine laboratory culture of VTEC strains in this project, as this easily available commercial medium has been reported to result in fast growth rates, a high cell yield, and levels of VT equivalent to those produced in Evans medium (Karmali *et al.*, 1985b).

It should be noted that laboratory culture is not the normal environment that these pathogenic organisms find during infection, when growth rates are often restricted by nutrient limitation and host defenses. In order to gain a true understanding of the role of toxin production in the pathogenesis of VTEC associated diseases, it will be necessary to use *in vitro* growth conditions that mimic the *in vivo* condition as closely as possible. Direct comparison of *in vitro* virulence gene expression to the *in vivo* condition must therefore be made with caution.

Figure 3.1 Growth characteristics of a batch culture of *E. coli* O157:H7 (ATCC 35150)

100 ml Penassay medium was inoculated with 0.5 ml of an overnight culture of *E. coli* O157:H7 (ATCC 35150) and incubated at 37°C in a Gallenkemp orbital shaker (250 rpm). At the time intervals shown, the optical density at 600nm (OD600) was monitored spectrophotometrically. The total cell count and mean cell volume of the culture were also determined using an electrical particle analyser (Coulter Counter) as described in section 2.7.5.



3.3 Biochemical characteristics

The performance of each strain in 21 biochemical tests was determined using the API20E identification system (Bio Mérieux, France) as described in section 2.8. The results obtained are shown in table 3.1. The API20E system is a standardised, miniaturised version of conventional physiological procedures for the identification of Enterobacteriaceae and other Gram negative bacteria. The pattern of reactions were converted into a numerical profile and identification made using a computer based profile recognition system. All profiles were identified as *E. coli*., however, comparison of individual strain reactions with the percentage of positive reactions normally observed for *E. coli* resulted in information on atypical biochemical characteristics.

E. coli K12 showed no atypical characteristics and was almost identical biochemically to *E. coli* C600(933J) and C600(933W), except that the latter two strains were unable to ferment melibiose. *E. coli* strains O26:H11 (E3787) and O26:H11 (NCTC 8781) also showed very similar profiles, both atypically being unable to ferment rhamnose (typically 87% positive). *E. coli* O26:H11 (NCTC 8781) however, also displayed an additional atypical characteristic, that of urease production (typically 1% positive). *E. coli* strains O157:H7 (ATCC 35150), E32511 (O157:H$^-$) and the Clinical Isolate all displayed identical profiles. Most notably, these strains were unable to ferment sorbitol (typically 90% positive). This atypical characteristic has been used as a strain marker to aid in the detection of *E. coli* O157:H7 in mixed flora on MacConkey agar containing D-sorbitol in place of lactose (SMAC), and is discussed in detail in section 1.8.2. Presumptive *E. coli* O157:H7 isolates on SMAC must be specifically typed through slide agglutination using O157 and H7 antisera. Subsequent use of a latex agglutination test for the identification of *E. coli* serogroup O157 and *E. coli* H antiserum H7 (section 2.9) identified the *E. coli* Clinical Isolate serologically as being O157:H7 (section 2.9). The Clinical Isolate was subsequently termed *E. coli* O157:H7 (CI).

Table 3.1 Biochemical characteristics of *Escherichia coli* strains using the API 20E identification system (Bio Merieux SA, France)

STRAIN	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
K12	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	+	+	+	-	+	-
O157:H7 (ATCC 35150)	+	-	+	+	-	-	-	-	+	-	-	+	+	-	-	+	+	+	-	+	-
O157:H7 (CI)	+	-	+	+	-	-	-	-	+	-	-	+	+	-	-	+	+	+	-	+	-
O26:H11 (NCTC 8781)	+	-	+	+	-	-	+	-	+	-	-	+	+	-	+	-	+	+	-	+	-
E3787	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	-	+	+	-	+	-
E32511	+	-	+	+	-	-	-	-	+	-	-	+	+	-	-	+	+	+	-	+	-
C600(933J)	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	+	-
C600(933W)	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	+	-

+ = positive reaction ; - = negative reaction

Key to table

Test	Reactions/Enzymes	Test	Reactions/Enzymes	Test	Reactions/Enzymes
ONPG	beta-galactosidase	TDA	tryptophan desaminase	SOR	sorbitol fermentation/oxidation
ADH	arginine dihydrolase	IND	indole production	RHA	rhamnose fermentation/oxidation
LDC	lysine decarboxylase	VP	acetoin production	SAC	sucrose fermentation/oxidation
ODC	ornithine decarboxylase	GEL	gelatinase	MEL	melibiose fermentation/oxidation
CIT	citrate utilisation	GLU	glucose fermentation/oxidation	AMY	amygdalin fermentation/oxidation
H_2S	H_2S production	MAN	mannitol fermentation/oxidation	ARA	arabinose fermentation/oxidation
URE	urease	INO	inositol fermentation/oxidation	OX	cytochrome-oxidase

To confirm that the above strains had not mutated with respect to biochemical characteristics during repetitive subculturing, API20E identification profiles were repeated on two further occasions at later stages of the project and found to be identical to those obtained in table 3.1. Additional studies (section 2.10), in which growth of the strains on purine/pyrimidine, amino acid and vitamin pools was examined (section 2.3.6), revealed that the strains associated with diarrhoeal disease were prototrophic and able to grow on minimal salts media.

3.4 Phage production and phage challenge

Production of VT1 and VT2 has been shown to result from infection by temperate bacteriophage carrying the structural genes for toxin production (section 1.4.1). Temperate bacteriophage, as well as undergoing a lytic cycle which causes bacterial lysis, may in certain circumstances, persist indefinitely in the host cell as an integral part of the host genome without phage production. This phenomenon is known as lysogeny. In order to determine/confirm the presence of lysogenic bacteriophage within the strains under investigation, phage lysates were prepared following addition of mitomycin C (1 μg ml^{-1}) to the culture medium (section 2.11.1). Mitomycin C is thought to cause phage induction by the mechanism discussed in detail in section 1.4.4.5. Such phage lysates were used to challenge lawns of the non-lysogenic *E. coli* strain DH1, as described in section 2.11.2.

Strains E3787, E32511, O157:H7 (ATCC 35150), C600(933J), C600(933W) and K12 contained lysogenic bacteriophage, as evidenced by production of zones of lysis on lawns of *E. coli* DH1 (table 3.2). The proportion of challenged cells that demonstrated lysogeny varied significantly with, for example, production of a zone of almost complete cell lysis by lysate from *E. coli* E3787 (i.e. few infected cells lysogenised), whilst other lysates produced turbid zones of lysis (i.e. large percentage of infected cells lysogenised). Strains O26:H11 (NCTC 8781) and O157:H7 (CI) showed no evidence of

DH1 cell lysis. There are several possible explanations for the latter observation. Perhaps these strains do not contain lysogenic bacteriophage, *E. coli* DH1 may not have been sensitive to the phage produced by these strains, the strains may be lysogenised with unstable or defective phage, or the conditions used for induction or detection of phage were not optimum. An increase in the concentration of mitomycin C to 10 μg ml^{-1} during phage lysate preparation from strains O26:H11 (NCTC 8781) and O157:H7 (CI) were also negative.

Table 3.2 A summary of the results obtained by mitomycin C induction and challenge of the strains indicated with lambda *vir*.

Strain	Phage production	Lysis with lambda *vir*
E3787	Yes	No
E32511	Yes	No
O157:H7 (ATCC 35150)	Yes	No
O157:H7 (CI)	No	No
O26:H11 (NCTC 8781)	No	No
C600(933J)	Yes	Yes
C600(933W)	Yes	Yes
K12	Yes	Yes

Culture supernatants after growth in the presence of mitomycin C (1 μg ml-1) were used to challenge lawns of *E. coli* DH1 to show presence of phage. In addition a lawn of each *E. coli* strain was challenged with a high titre lamda *vir* lysate (2×10^{10} PFU ml^{-1}) and plates examined for bacterial cell lysis.

At the same time, lawns of all strains were prepared and challenged with a lysate containing the bacteriophage lambda *vir* (table 2.3). A temperate bacteriophage maintains the lysogenic state by producing a repressor, specified by the cI gene, which binds to operator sequences of the phage DNA, preventing expression of lytic functions. The cI repressor is also responsible for immunity to exogenous infection, preventing infecting phage DNA from replicating. Lambda *vir* is a virulent (*vir*) mutant with a mutation in the operator binding site for the cI repressor, resulting in a constitutive lytic

cycle. Once inside the cell, the lambda *vir* DNA does not recognise the cI repressor produced by the lysogenised phage genome, replication of the *vir* phage genome and subsequent cell lysis then occurs. It was observed that lambda *vir* caused lysis on lawns of *E. coli* strains K12, DH1, C600(933J) and C600(933W) but no lysis of the wild type strains E3787, E32511, O157:H7 (ATCC 35150), O157:H7 (CI) and O26:H11 (NCTC 8781), (table 3.2). Challenge of bacterial lawns with a freshly prepared high titre lambda *vir* lysate (section 2.11.3), with a phage titre of 2×10^{10} PFU ml^{-1} (section 2.11.4), gave identical results. The reason for this is unclear but would imply inability of the phage to enter these strains for some reason. This may be due to alteration/modification of the phage receptors on the cell surface of these strains, leading to bacteriophage resistance, or inability of the phage DNA to enter these cells. Alternatively, previous infection with phage carrying genes that led to the conversion of the surface antigens to prevent further adsorption of phage could have occurred. This would eliminate waste of phage on lysogenised cells. Investigation as to the reason for inability of lambda *vir* to cause lysis in these strains would require substantial investigation and as this was not an aim of this thesis, was not pursued.

3.5 Gene probing with polynucleotide probes

As mentioned in section 3.1, production of VT1 by strain E3787 and VT2 by strain E32511 has been reported previously (Willshaw *et al.*, 1987). However, it was necessary to confirm that the received strains possessed the published genotypes and to determine the presence/type of VT gene sequences in the other isolates. At this time, there had been no publications on the existance of VT's other than VT1, VT2 and VTe (table 1.1); synthetic oligonucleotide probes (table 1.7) or VT-specific oligonucleotide primers (table 1.8) to detect VT gene sequences. Cloned DNA fragments had, however, been used as gene probes to identify and distinguish those strains carrying gene sequences for VT1 and/or VT2 (section 1.8.8.1) and two strains (table 3.3) were provided by Bernard Rowe, Central Public Health Laboratory, London, for the preparation of probe

Table 3.3 DNA probes for VT1 and VT2 gene sequences

Strain	VT production	Plasmid carried	Vector plasmid	Inserted DNA fragment	Probe fragment	Reference[b]
60R746						
E. coli K12 F⁻ prototrophic	No	NTP705	pACYC177	0.75 Kb *Hin*cII	VT1 0.75 Kb *Hin*cII	(1)
60R363						
E. coli K12 F⁻ prototrophic	Yes	NTP707	pACYC184	4.7 Kb *Eco*RI	VT2 0.85 Kb *Sma*I-*Pst*I	(2)

[b] (1) Willshaw *et al.* (1985)
(2) Willshaw *et al.* (1987)

fragments for the detection of VT1 and VT2 genes. Strain 60R746 carries the recombinant plasmid NTP705 which has a 0.75 Kb *Hinc*II fragment containing sequences of the B subunit gene of VT1, inserted into the ampicillin resistance (Ap^r) gene of vector plasmid pACYC177. Strain 60R363 contains the recombinant plasmid NTP707 which carries a 4.7 Kb *Eco*RI fragment encoding the VT2 operon, inserted into the chloramphenicol resistance (Cm^r) gene of vector plasmid pACYC184 (figure 3.2). Double digestion of plasmid NTP707 with restriction enzymes *Sma*I and *Pst*I or *Ava*I and *Pst*I generates a fragment of 0.85 Kb containing sequences of the A subunit gene of VT2. Digestion with the latter two enzymes however, produces additional DNA fragments and therefore, *Sma*I-*Pst*I digestion was chosen, it being technically easier to subsequently isolate the required fragment.

3.5.1 Preparation of VT1 and VT2 probe fragments

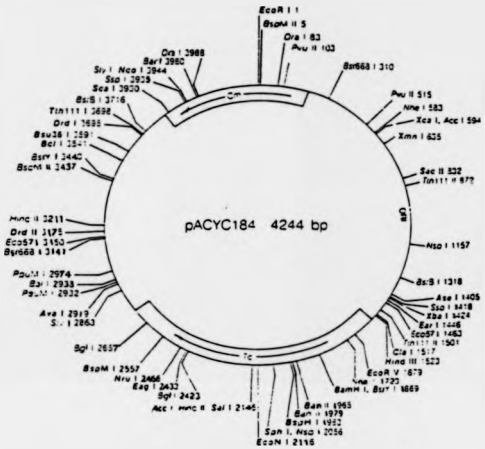
Large scale isolation of recombinant plasmids NTP705 and NTP707 was performed as described in section 2.13.1(b). To ensure maintenance of plasmids during preparation, strains 60R746 and 60R363 were cultured in LB containing the antibiotics, kanamycin (Kn) and tetracycline (Tc) respectively, at concentrations stipulated in section 2.4. To obtain the DNA probe fragments, the recombinant plasmids were restricted with the appropriate enzymes (table 3.3) and the digests run on a 1% (w/v) low gelling temperature agarose (BRL) gel (section 2.15). The VT1 and VT2 probe fragments (figure 3.3), were then excised under short-wavelength UV and labelled to high specific activity, without prior purification from the gel slice, as described in section 2.17.

3.5.2 Probing of chromosomal and plasmid DNA

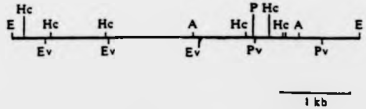
Large scale isolation of chromosomal and plasmid DNA from each strain was performed as described in sections 2.12(b) and 2.13.1(b) respectively. Samples of plasmid DNA were electrophoresed through 1% (w/v) agarose gels (section 2.15) whilst

Figure 3.2 **Restriction enzyme maps of :- (a) vector plasmid pACYC184 and (b) a 4.7 Kb *Eco*RI fragment containing the VT2 operon**

(a)



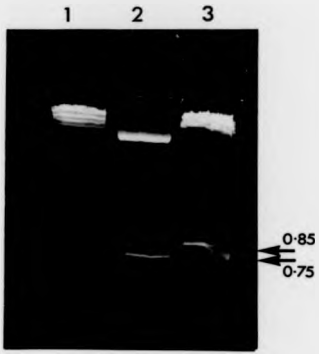
(b)



The 4.7 Kb *Eco*RI fragment cloned from ϕ32511 (VT2) was inserted into *Eco*RI digested vector plasmid pACYC184 to form recombinant plasmid NTP707 (Willshaw *et al.*, 1987).

A, *Ava*I; E, *Eco*RI; Ev, *Eco*RV; Hc, *Hinc*II; P, *Pst*I; Pv, *Pvu*II.

Figure 3.3 DNA probe fragments for VT1 and VT2



1% (w/v) low gelling temperature agarose (BRL) gel run in Tris-borate buffer.

Key to tracks

1 : Phage lambda DNA digested with *Hind*III. This generated fragment sizes : 23.17, 9.46, 6.75. 4.26, 2.20, 1.92 & 0.58 Kb.

2 : Plasmid NTP705 digested with *Hinc*II.

3 : Plasmid NTP707 digested with *Sma*I and *Pst*I.

Arrowed fragments are those used as VT1 (0.75 Kb) and VT2 (0.85 Kb) probes.

chromosomal DNA samples were digested with *Eco*RI (section 2.14) and subjected to electrophoresis through 0.7% (w/v) agarose gels. Duplicate gels were then Southern blotted (section 2.18), hybridised with either the VT1 or the VT2 labelled probes (section 2.19) and the filters examined by autoradiography (section 2.20). The A and B subunits of VT1 and VT2 show 57% and 60% overall nucleotide sequence homology respectively (table 1.4), however, no cross-hybridisation between the probes was observed after stringent washes in 0.1 x SSC, 0.1% (w/v) SDS, as detailed in section 2.19. A summary of the individual strain reactions with the labelled gene probes is shown in table 3.4.

Table 3.4 **Summary of the results obtained by probing chromosomal DNA of the strains indicated with ^{32}P-dCTP labelled VT1 and VT2 DNA probes**

Strain	VT1 probe	Size of *Eco*RI fragment (Kb)	VT2 probe	Size of *Eco*RI fragment (Kb)
E3787	+	6.5	-	-
E32511	-	-	+	4.9 & 4.3
O157:H7 (ATCC 35150)	+	6.5	+	4.3
O157:H7 (CI)	-	-	+	13.5 & 5.8
O26:H11 (NCTC 8781)	-	-	-	-
C600(933J)	+	6.4	-	-
C600(933W)	-	-	+	4.4
K12	-	-	-	-
S.dysenteriae type 1*	+	4.5	-	-

*Shiga toxin producer.

\+ = Hybridisation after stringent washing in 0.1 x SSC, 0.1% (w/v) SDS.

\- = No hybridisation after stringent washing in 0.1 x SSC, 0.1% (w/v) SDS.

There has been no published description of VT genes occurring on the natural plasmids found in some VTEC strains, similarily, Southern blots of plasmid DNA isolated from the strains examined in this thesis showed no hybridisation with either the VT1 or the VT2 labelled DNA probes (data not shown). Figure 3.4 shows photographs of the electrophoresed *Eco*RI restricted chromosomal DNA of the strains tested and the corresponding autoradiographs obtained upon probing with the ^{32}P-dCTP labelled VT1 probe. Figure 3.5 shows the corresponding autoradiographs obtained after probing with the ^{32}P-dCTP labelled VT2 probe. From the results it can be confirmed that strains E3787 and C600(933J) carry VT1 gene sequences and that strains E32511 and C600(933W) carry gene sequences homologous to VT2. In addition O157:H7 (ATCC 35150) was found to contain VT1 and VT2 gene sequences whereas O157:H7 (CI) contained only VT2 gene sequences. A single *Eco*RI fragment of *Shigella dysenteriae* type 1 chromosomal DNA was seen to hybridise with the VT1 probe. This was not unexpected since *S. dysenteriae* type 1 contains the genes for Shiga toxin (ShT), which is almost identical to VT1, with only three nucleotide differences occurring in the A subunit (section 1.4.3.1). Strains O26:H11 (NCTC 8781) and K12 showed no homology with either of the DNA probes and as *E. coli* O26:H11 also did not appear to contain mitomycin C inducible phage (section 3.4), it is possible that loss of this phage, with the associated VT production, may have occurred during storage. It can be seen from the autoradiographs that two *Eco*RI fragments in the chromosomal DNA of E32511 and O157:H7 (CI) hybridised with the VT2 gene probe under stringent conditions. This implied that these strains have two copies of VT2-related genes, since none of the *slt* operons sequenced to date have contained an *Eco*RI site within or between the structural genes for the A and B polypeptides. E32511 has previously been documented to only produce VT2 (Scotland *et al.*, 1985; Head *et al.*, 1988a). Production of a VT2-variant toxin by E32511 may account for the discrepancies reported concerning the physical properties of the VT2 toxin purified from this strain (Head *et al.*, 1988a), compared with that purified from the recombinant version in pEB1 (Downes *et al.*, 1988). Antigenic differences have also been reported when antisera from these two

strains were used in neutralisation assays (Head *et al.*, 1988b). Also Dickie *et al.* (1989) found that antisera from VT2 producing strain 3657 (serogroup O157:H7) and K12 (pEB1) could not completely neutralise the VT activity of E32511. It would have been necessary to clone and sequence these operons to determine their identity and relationship with VT2 and as this was not the aim of the project, was not pursued further. Oku *et al.* (1989) published evidence of a VT2 variant toxin, suspected as being VTe, being produced by an *E. coli* O91:H21 strain isolated from a patient with HUS. The subsequent cloning and sequencing of genes from *E. coli* O91:H21 identified two distinct genes, *vtx*2ha and *vtx*2hb, with almost identical nucleotide sequences, that were capable of producing a VT similar to VT2 (section 1.4.3.4). This was the first published demonstration of multiple copies of toxin genes related to VT2 within a single strain. Schmitt *et al.* (1991) also reported that E32511 contained two copies of VT2-related genes and published the nucleotide sequences of the two different operons. One was determined to be that of *slt*-II while the other, termed *slt*-IIc in essence contained A subunit sequences from *slt*-II and B subunit sequences from *vtx*2ha (table 1.6). In Schmitts study, DNA from E32511 digested with *Eco*RI gave two bands of approximately 4.8 and 5.5 Kb which showed hybridisation with the VT2 probe. Willshaw *et al.* (1985) reported the cloning of VT2 (E32511) from a 4.7 Kb *Eco*RI fragment derived from phage DNA of E32511, whereas Rietra *et al.* (1989) reported that a 5.0 Kb *Eco*RI fragment from phage DNA of E32511 hybridised with VT2-specific sequences. The values obtained in this study (table 3.4) are slightly lower, being 4.9 and 4.3 Kb. Based on Schmitts results it was concluded that the 4.3 Kb *Eco*RI fragment observed in figure 3.5 carries the *slt*-II operon whilst the 4.9 Kb *Eco*RI fragment carries the *slt*-IIc operon. The *Eco*RI fragment in *E. coli* O157:H7 (ATCC 35150) that hybridised with the VT2 probe, comigrated with the 4.3 Kb *Eco*RI fragment of E32511 (data not shown) and it is proposed therefore that this strain, in addition to VT1, also produces VT2.

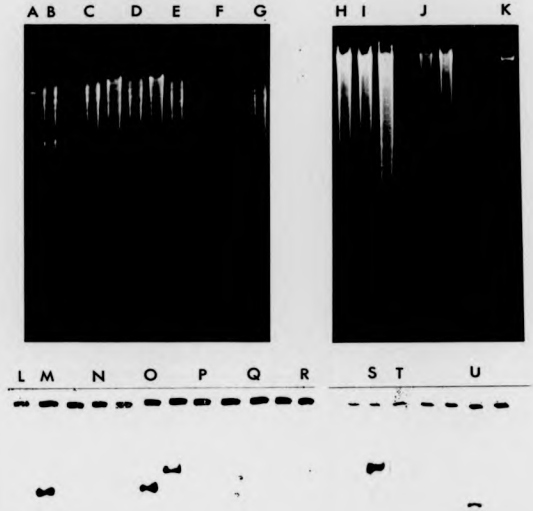

A B C D E F G
H I J K
L M N O P Q R
S T U

Figure 3.4 Examination of chromosomal DNA of *E. coli* strains for VT1 gene sequences

Chromosomal DNA of the strains indicated was restricted with *Eco*RI, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. The resulting blot was probed with a ^{32}P-dCTP labelled VT1 DNA probe cloned from the phage DNA of *E. coli* strain H19 (serotype O26:H11). Chromosomal DNA from *S. dysenteriae* type 1 was also probed.

Key to tracks

A & K: Phage lambda DNA restricted with *Hind*III.

B to J: E3787, E32511, O157:H7 (ATCC 35150), O157:H7 (CI), O26:H11 (NCTC 8781), K12, C600(933J), C600(933W) & *S. dysenteriae* type 1 chromosomal DNA respectively - restricted with *Eco*RI.

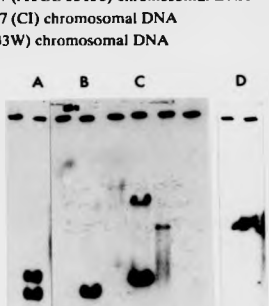
Tracks L to U are autoradiographs of the corresponding tracks A to J probed with the ^{32}P-dCTP labelled VT1 probe.

Figure 3.5 Examination of chromosomal DNA of *E. coli* strains for VT2 gene sequences

A Southern blot of *Eco*RI restricted chromosomal DNA of the strains shown in figure 3.4 was probed with a ^{32}P-dCTP labelled VT2 DNA probe cloned from the phage DNA of *E. coli* strain E32511 (serotype O157:H$^-$). Only tracks showing hybridisation are shown. All the other strains showed no evidence of hybridisation.

Key to tracks

A : E32511 chromosomal DNA
B : O157:H7 (ATCC 35150) chromosomal DNA
C : O157:H7 (CI) chromosomal DNA
D : C600(933W) chromosomal DNA



Most prophages are integrated at fixed locations on the bacterial chromosome. The VT1 *Eco*RI fragments of strains E3787, C600(933J) and O157:H7 (ATCC 35150) are similar in size and presumably, this is due to the integration of the VT1 carrying phage at identical sites in the bacterial chromosomes of these strains. Similarily, the VT2 carrying phage would appear to have integrated at identical sites in the chromosomes of *E. coli* strains E32511, C600(933W) and O157:H7 (ATCC 35150). Neither of the *Eco*RI fragments that hybridised with the VT2 probe in O157:H7 (CI), comigrated with the *Eco*RI fragments in E32511 and the identity of the VT2-related operons in this strain remain to be characterised. *E. coli* O157:H7 (CI) did not appear to contain mitomycin C inducible phage (section 3.4), and it is possible that this strain may have obtained VT2-related sequences by a method of gene transfer such as conjugation. Transfer of genes coding for VTe from a field isolate to a K12 strain by conjugation has been reported (Williams Smith *et al.*, 1983).

Studies have shown that *E. coli* O157:H7 strains containing only VT2 genes are more frequently associated with HUS and TTP than strains containing VT1 or both VT1 and VT2 (Ostroff *et al.*, 1989; Tarr *et al.*, 1989). Scotland *et al.* (1987) also noted that strains producing VT2 alone were most frequently associated with HUS. The importance of multiple copies of *slt*-II operons in strains of *E. coli* associated with disease remains to be determined. So far there has been no evidence of multiple copies of VT1 related genes in a single strain.

3.6 Determination of VT production using the ricin assay

The ricin assay was used to determine which strains were capable of producing active toxin. This assay makes use of the fact that both ricin and VT's have exactly the same mode and site of action (sections 1.3.3 & 1.3.3.2). Treatment of eukaryotic ribosomes with these toxins results in cleavage of a specific N-glycosidic bond at adenine residue 4324 in 28S ribosomal RNA (rRNA). Addition of aniline to the isolated RNA causes

release of a distinctive fragment of 400 nucleotides which can be easily visualised on RNA gels.

Cell pellets were treated with polymyxin B (section 2.21.1), as polymyxin B has previously been found to be useful in extracting high titres of intracellular VT from cell pellets of VTEC (Karmali *et al.*, 1985b). Polymyxin B extracts were reduced with BME (section 2.21.1) to ensure production of catalytically active A1 fragments and used to treat yeast ribosomes (section 2.21.2) as described in section 2.21.2.1. As a positive control, yeast ribosomes were also incubated in the presence of recombinant ricin A chain, a gift from Dr. Martin Hartley, Warwick University. The recombinant protein contains an N-terminal methionine prior to isoleucine, the N-terminal amino acid *in planta*. Apart from this it is identical to the native, plant purified A chain in primary sequence. RNA was extracted from toxin-treated ribosomes (section 2.21.2) and treated with aniline as described in section 2.21.2.2. Toxin treated rRNA, before and after aniline treatment was electrophoresed through a 1.2% (w/v) agarose/50% (v/v) formamide gel and the RNA visualised after soaking in ethidium bromide (section 2.21.2.3).

3.6.1 Requirement of a β-mercaptoethanol incubation step

In an initial experiment, yeast ribosomes were treated with E3787 and E32511 polymyxin B extracts to which no BME had been added. The distinctive 400 nucleotide fragment released upon aniline treatment was present in both ricin and E32511 treated RNA tracks, but was absent from the E3787 treated RNA track (gel not shown). When BME was included in the reaction mix (2 μl of 100 mM stock), identical results were obtained (figure 3.6). In another experiment, in addition to inclusion of BME in the reaction mix (2 μl of 100 mM stock), the polymyxin B cell extracts were also preincubated in BME (10 μl extract + 1 μl 100 mM BME, 30^{0}C, 1 hour). On this occasion (figure 3.7), E3787 polymyxin B cell extracts contained active toxin, as

Figure 3.6 **Analysis of yeast ribosomal RNA (rRNA) treated with *E. coli* E3787 and E32511 polymyxin B cell extracts and β-mercaptoethanol (BME) in the reaction mixture.**

All samples, except for the ricin control had 2 μl of 100 mM BME included in the reaction mixture as described in materials and methods. Electrophoresis of RNA was performed on a 1.2% (w/v) agarose, 50% (v/v) formamide gel. The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer, stained in 2 μg ml^{-1} ethidium bromide for 20 minutes and destained in distilled water for 10 minutes.

Key to tracks

A : Yeast rRNA treated with recombinant ricin A chain (+ aniline).

B : Yeast rRNA treated with recombinant ricin A chain (- aniline).

C : Yeast rRNA treated with *E. coli* E3787 polymyxin B cell extract (+ aniline).

D : Yeast rRNA treated with *E. coli* E3787 polymyxin B cell extract (- aniline).

E : Yeast rRNA treated with *E. coli* E32511 polymyxin B cell extract (+ aniline).

F : Yeast rRNA treated with *E. coli* E32511 polymyxin B cell extract (- aniline).

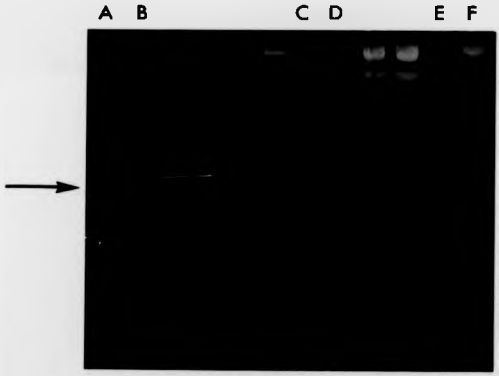


Figure 3.7 **Analysis of yeast ribosomal RNA treated with *E. coli* E3787 and E32511 polymyxin B cell extracts, pre-incubated with BME in addition to BME in the reaction mixture.**

Polymyxin B cell extracts were pre-incubated with 1 μl of 100 mM BME for 1 hour at 30^{0}C in addition to the 2 μl of 100 mM BME included in the reaction mixture, except for the ricin control. Electrophoresis of RNA was performed on a 1.2% (w/v) agarose, 50% (v/v) formamide gel. The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer, stained in 2 μg ml^{-1} ethidium bromide for 30 minutes and destained in distilled water for 10 minutes.

Key to tracks

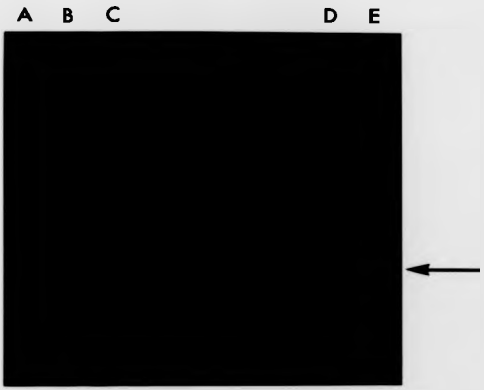
A : Yeast rRNA treated with recombinant ricin A chain (+ aniline).

B : Yeast rRNA treated with *E. coli* E3787 polymyxin B cell extract (- aniline).

C : Yeast rRNA treated with *E. coli* E3787 polymyxin B cell extract (+ aniline).

D : Yeast rRNA treated with *E. coli* E32511 polymyxin B cell extract (- aniline).

E : Yeast rRNA treated with *E. coli* E32511 polymyxin B cell extract (+ aniline).



evidenced by presence of aniline releasable fragment (arrowed in figures 3.6 and 3.7). These observations imply that strain E32511, which contains *slt*-II and *slt*-IIc operons (section 3.5.2), produces toxin(s) that does not require reduction to be catalytically active. Either the A subunit possesses N-glycosidase activity in the non-reduced form or it is very unstable and readily dissociates to form the active A1 fragment. VT1 produced by strain E3787, on the other hand, requires reduction with BME and furthermore prolonged exposure to this reducing agent (1 hour, 30^{o}C was found to be successful) before displaying N-glycosidase activity. Production of toxin which is readily active (VT2 by E32511) as opposed to a toxin which requires relatively lengthy reduction before displaying activity has important implications on the relative abilities of such organisms to cause disease *in vivo*, the former could presumably lead to a more sudden onset of symptoms.

3.6.2 Determination of toxin production by bacterial strains

The ricin assay was used as described above (section 3.6), to determine which strains, in addition to *E. coli* E3787 and E32511 were capable of producing active toxin. Polymyxin B cell extracts were preincubated in BME to ensure that no false negatives were obtained (section 3.6.1). The resulting gel is shown in figure 3.8. All strains were seen to produce active toxin except *E. coli* strains O26:H11 (NCTC 8781) and K12. This corroborates the gene probing data obtained in section 3.5.2.

3.6.3 Comparison of *E. coli* E3787 and E32511 polymyxin B and PBS cell extracts in the ricin assay

Polymyxin B and PBS cell extracts were prepared from strains E3787 and E32511 as described in section 2.21.1 except that the cell pellets for PBS extraction were resuspended in 1 ml of sterile PBS instead of polymyxin B. Yeast rRNA was challenged with these extracts and the resulting gel is shown in figure 3.9. It can be seen that

Figure 3.8 Analysis of yeast ribosomal RNA treated with polymyxin B cell extracts of *E. coli* strains.

Electrophoresis of RNA was performed on a 1.2% (w/v) agarose, 50% (v/v) formamide gel. The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer, stained in 2 $\mu g\ ml^{-1}$ ethidium bromide for 15 minutes and destained in distilled water for 30 minutes.

Key to tracks

A : Yeast rRNA treated with recombinant ricin A chain (- aniline).
B : Yeast rRNA treated with recombinant ricin A chain (+ aniline).
C : Yeast rRNA treated with O157:H7 (ATCC 35150) cell extract (- aniline).
D : Yeast rRNA treated with O157:H7 (ATCC 35150) cell extract (+ aniline).
E : Yeast rRNA treated with O157:H7 (CI) cell extract (- aniline).
F : Yeast rRNA treated with O157:H7 (CI) cell extract (+ aniline).
G : Yeast rRNA treated with O26:H11 (NCTC 8781) cell extract (- aniline).
H : Yeast rRNA treated with O26:H11 (NCTC 8781) cell extract (+ aniline).
I : Yeast rRNA treated with K12 cell extract (- aniline).
J : Yeast rRNA treated with K12 cell extract (+ aniline).
K : Yeast rRNA treated with C600(933J) cell extract (- aniline).
L : Yeast rRNA treated with C600(933J) cell extract (+ aniline).
M : Yeast rRNA treated with C600(933W) cell extract (- aniline).
N : Yeast rRNA treated with C600(933W) cell extract (+ aniline).

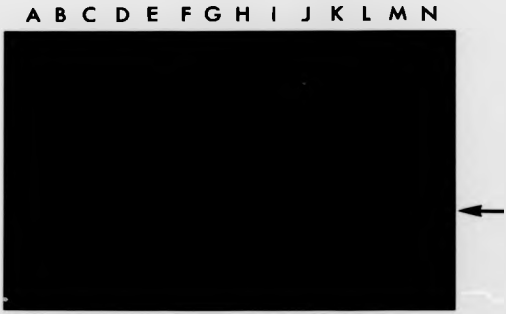
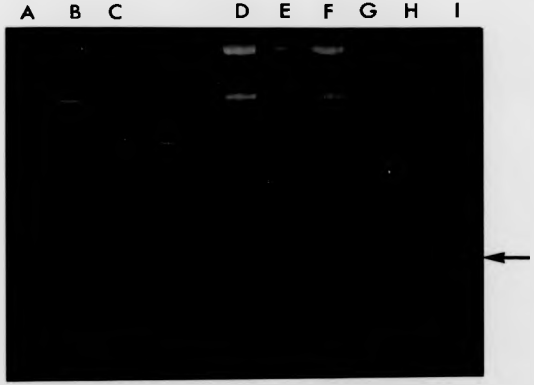


Figure 3.9 **Analysis of yeast ribosomal RNA treated with either phosphate buffered saline (PBS) or polymyxin B cell extracts of *E. coli* strains E3787 and E32511.**

Electrophoresis of RNA was performed on a 1.2% (w/v) agarose, 50% (v/v) formamide gel. The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer, stained in 2 $\mu g\ ml^{-1}$ ethidium bromide for 30 minutes and destained in distilled water for 10 minutes.

Key to tracks

A : Yeast rRNA treated with recombinant ricin A chain (+ aniline).
B : Yeast rRNA treated with E3787 polymyxin B cell extract (- aniline).
C : Yeast rRNA treated with E3787 polymyxin B cell extract (+ aniline).
D : Yeast rRNA treated with E3787 PBS cell extract (- aniline).
E : Yeast rRNA treated with E3787 PBS cell extract (+ aniline).
F : Yeast rRNA treated with E32511 polymyxin B cell extract (- aniline).
G : Yeast rRNA treated with E32511 polymyxin B cell extract (+ aniline).
H : Yeast rRNA treated with E32511 PBS cell extract (- aniline).
I : Yeast rRNA treated with E32511 PBS cell extract (+ aniline).



polymyxin B cell extracts of both strains contain active toxin (tracks C & G) whereas only strain E32511 has active toxin in PBS treated cell extracts (track I). The presence of VT in PBS treated cell extracts is compatible with the concept of a leaky outer membrane and, moreover, it would appear that only VT2 and not VT1 is able to leak from the periplasm. PBS and polymyxin B cell extracts from both strains had been prepared from late exponential phase cultures and it is unlikely that the appearance of toxin in PBS extracts was due to bacterial autolysis. These findings corroborate earlier studies by Karmali *et al.* (1985b) who proposed that VT1 predominates in cell lysates with VT2 present in much higher titres than VT1 in culture supernatants. Both findings imply significant differences in the secretion and localisation of the two different toxins and furthermore, the demonstration that VT2 readily leaks whilst VT1 does not may be important in the pathogenesis of VT2 associated diseases. Secretion of an active (section 3.6.1) toxin into the surrounding environment during growth could lead to a sudden onset of symptoms and additionally reduce the available time in which the body could raise a protective immune reponse. By this time the damage may have been done and indeed this hypothesis may be reflected in the findings of a number of groups which have shown that strains producing VT2 alone are most frequently associated with HUS (section 3.5.2).

3.7 Protein profiles of *E. coli* O157:H7 (ATCC 35150) during growth on Penassay broth

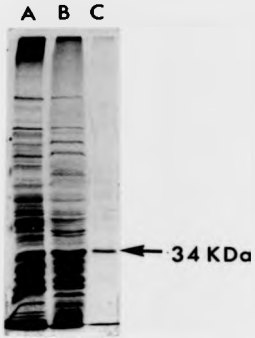
Earlier in the project, cell free extracts and culture supernatant samples were prepared at known time intervals during growth of *E. coli* O157:H7 (ATCC 35150) on Penassay broth (sections 2.23 and 2.24 respectively). A protein concentration step was required in the preparation of culture supernatant samples and ammonium sulphate precipitation was initially used (section 2.24.1). It was found necessary however, to concentrate large volumes of supernatant, (typically 2 litres), due to protein losses during procedural steps. An Amicon Micro-Ultrafiltration System (Amicon Corp.),

incorporating a 10 KDa cut-off filter was found to be more practical (section 2.24.2). Prior to concentration in the latter, it was necessary to dialyse culture supernatant samples against 10 mM Tris-HCl, pH 7.0 (section 2.24.2). This removed substances (presumably medium salts) which were found to interfere with subsequent protein staining.

The protein concentration of each sample was determined using the Bio-Rad protein assay as described in section 2.22. Cell extract and supernatant samples adjusted to contain the same amount of protein (as indicated), were then analysed by 11% (w/v) SDS-PAGE as described in section 2.25.1 with protein molecular weight (MW) markers of size range 14.4-94.0 KDa (section 2.29). The proteins were visualised by silver staining (section 2.25.2), destained if necessary (section 2.25.3) and photographed (section 2.26).

Figure 3.10 shows a SDS polyacrylamide gel of cell extract and concentrated supernatant samples (tracks A and B respectively), of a stationary phase culture of *E. coli* O157:H7 (ATCC 35150) grown on Penassay broth. In tracks A and B it can be seen that a protein (arrowed), estimated to have a MW of approximately 34 KDa, is present which comigrates with purified O157:H7 VT (provided by Sue Colby, Warwick University). This is thought to correspond to the A subunit of the toxin, with the much smaller B subunit comigrating with the dye front. Later in the project, gene probing studies demonstrated that strain O157:H7 contained both VT1 and VT2 gene sequences (section 3.5). Purely on a MW basis, the purified protein was tentatively identified as the A subunit of VT2, as the A subunit of VT1 is slightly smaller (table 1.2). Furthermore it was shown in section 3.6.3 that VT1 appears to be located predominately intracellularly, whereas VT2 is present in much higher titres than VT1 in culture supernatants. As the purified VT had been isolated from the supernatant of a culture of O157:H7 (ATCC 35150), it is more likely that VT2 rather than VT1 was purified. Confirmation of the identities of the proteins corresponding to the VT1 and

Figure 3.10 **SDS-PAGE of cell extract and concentrated supernatant samples of a stationary phase culture of *E. coli* O157:H7 (ATCC 35150) grown on Penassay broth.**



Key to tracks

A : 6 hour cell extract - 45 μg protein.

B : 6 hour concentrated supernatant - 90 μg protein.

C : Purified VT from *E. coli* O157:H7 (ATCC 35150) - 10 μl (unknown concentration)

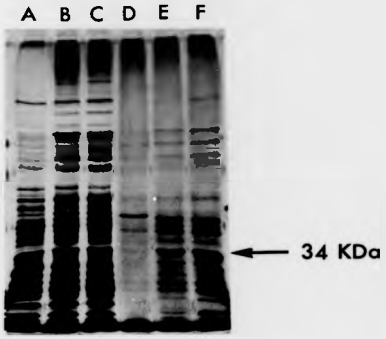
VT2 A subunits would have required Western blotting with specific antibodies to each, unfortunately these were not available at the time.

Figure 3.11 shows a SDS polyacrylamide gel of cell extract (tracks A to C) and concentrated supernatant (tracks D to F) samples of *E. coli* O157:H7 (ATCC 35150), prepared at the culture growth times indicated. It would appear that as late exponential/early stationary phase is reached, there is a sudden appearance in the supernatant of the protein thought to correspond to the VT2 A subunit. Production of the latter continues into the stationary phase, as evidenced by the denser protein band in track F. The ability of VT2 to readily leak across the outer membrane has already been discussed (section 3.6.2). Furthermore, it would appear (figure 3.11), that VT2 leaks significantly from the cells only as stationary phase of growth is approached and not earlier in the growth cycle. This may purely be the result of accumulation of VT2 to such an extent that leakage occurs at this point of growth. Alternatively, VT2 may have a role other than as a virulence determinant which as yet has not been recognised, for example, the aquisition of a specific nutrient which may have become limiting as the medium becomes spent.

3.8 Pulse labelling of *E. coli* O157:H7 (ATCC 35150) with ^{35}S-methionine.

In an attempt to more clearly define at what time during growth VT2 is produced, pulse-labelling experiments with ^{35}S-methionine were performed (section 2.28). Studies to determine the kinetics of ^{35}S-methionine incorporation by growing cultures of *E. coli* O157:H7 on Penassay containing 1μCi ml^{-1} final concentration of ^{35}S-methionine (section 2.27), revealed an initial lag in incorporation (figure 3.12). Similar kinetics of incorporation were observed when O157:H7 was grown on Penassay containing 2μCi ml^{-1} final concentration of ^{35}S-methionine (data not shown). As a final concentration of 1μCi ml^{-1} was equivalent to 7.69 x 10^{-2} nM methionine, L-methionine (7.7μl of 10μM) was added 1 hour prior to addition of the ^{35}S-methionine

Figure 3.11 **SDS-PAGE of cell extract and concentrated supernatant samples prepared during growth of *E. coli* O157:H7 (ATCC 35150) on Penassay broth.**

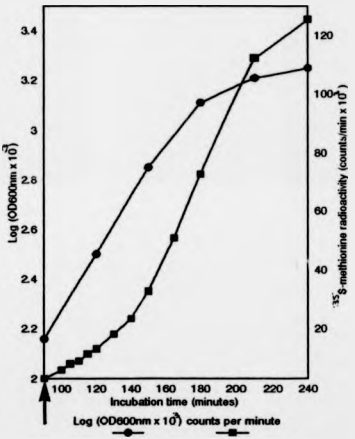


Key to tracks

A : 2 hour cell extract - 55 μg protein.
B : 4 hour cell extract - 55 μg protein.
C : 6 hour cell extract - 55 μg protein.
D : 2 hour concentrated supernatant - 55 μg protein.
E : 4 hour concentrated supernatant - 55 μg protein.
F : 6 hour concentrated supernatant - 55 μg protein.

to ensure there was no lag in the incorporation of label during pulse-labelling (section 2.28). In figure 3.12 it can be concluded that the available ^{35}S-methionine concentration did not become limiting during the time course of the experiment as the radioactive level of the precipitated cells did not plateau, except as the cells reached early stationary phase. To ensure excess available ^{35}S-methionine however, 3μCi ml^{-1} were added in the pulse labelling experiments.

Figure 3.12 **^{35}S-methionine incorporation during growth of *E. coli* O157:H7 (ATCC 35150) on Penassay broth**



At the time arrowed, ^{35}S-methionine was added to a growing culture of *E. coli* O157:H7 (ATCC 35150) to a final concentration of 1 μCi ml^{-1}. Assays were performed as described in materials and methods.

Figures 3.13 and 3.14 show silver stained SDS polyacrylamide gels of cell extract and supernatant samples prepared at known time intervals after a 10 min pulse with ^{35}S-methionine and the corresponding autoradiographs after 22 days exposure at room temperature (section 2.28). Samples in figures 3.13 and 3.14 had been adjusted to contain the same amount of protein or counts respectively as indicated. Once again it appeared that VT2 was only produced extracellularly as the culture reached early stationary phase (tracks H & I, figures 3.13 & 3.14). VT2 synthesis appeared to occur throughout growth but was slightly greater at 2 hours growth (track M, figures 3.14 & 3.15), when the culture was growing exponentially. Labelling of the VT2 toxin A subunit with ^{35}S-methionine was poor, labelled toxin A subunit could not be seen in the supernatant fractions of the autoradiographs and longer exposure to the ^{35}S-labelled material was required. Another factor that may have contributed to the poor degree of toxin labelling is that only five of the 296 amino acids that correspond to the processed A subunit of VT2 are methionine (Jackson *et al.*, 1987b). It can however be concluded from these results that VT2 synthesis occurred throughout growth and that the toxin accumulated intracellularly until stationary phase was approached.

3.9 Summary and overview.

The growth characteristics and nutritional requirements of the *E. coli* strains used in the project have been defined. Additionally the toxin genotype of each strain was determined/confirmed and the possession of lysogenic phage and the ability to produce active toxin was demonstrated. Differences in the secretion and localisation of VT1 and VT2 within *E. coli* E3787 and E32511 respectively have been shown. Whilst VT1 was strongly cell associated, VT2 readily leaked into the extracellular medium during growth. Furthermore VT2 was shown to possess N-glycosidase activity much more readily than VT1 and was produced into the supernatant only as the bacterial culture reached stationary phase.


A B C D E F G H I J
K L M N O P Q R S T

Figure 3.13 **SDS-PAGE of cell extract and concentrated supernatant samples of *E. coli* O157:H7 (ATCC 35150) grown on Penassay broth and pulsed with ^{35}S-methionine.**

Samples were adjusted to contain the same amount of protein per track as indicated.

Key to tracks

A&J: C_{14} labelled protein markers (200, 92.5, 69, 46, 30 & 14.3 KDa).
B : 1 hour cell extract - 16 μg protein.
C : 2 hour cell extract - 16 μg protein.
D : 4 hour cell extract - 16 μg protein.
E : 6 hour cell extract - 16 μg protein.
F : 1 hour concentrated supernatant - 32 μg protein.
G : 2 hour concentrated supernatant - 32 μg protein.
H : 4 hour concentrated supernatant - 32 μg protein.
I : 6 hour concentrated supernatant - 32 μg protein.

Tracks K to T are autoradiographs of the corresponding tracks A to J showing ^{35}S-methionine labelled proteins in the sample.

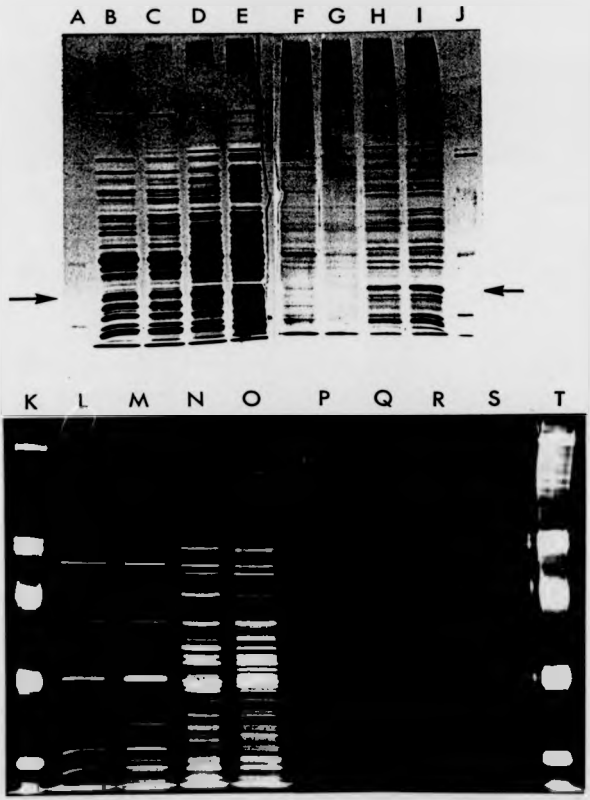

A B C D E F G H I J
K L M N O P Q R S T

Figure 3.14 SDS-PAGE of cell extract and concentrated supernatant samples of *E. coli* O157:H7 (ATCC 35150) grown on Penassay broth and pulsed with ^{35}S-methionine.

Samples were adjusted to contain the same counts min^{-1} per track as indicated.

Key to tracks

A&J: C_{14} labelled protein markers (200, 92.5, 69, 46, 30 & 14.3 KDa).
B : 1 hour cell extract - 12377 counts min^{-1}.
C : 2 hour cell extract - 12380 counts min^{-1}.
D : 4 hour cell extract - 12351 counts min^{-1}.
E : 6 hour cell extract - 12386 counts min^{-1}.
F : 1 hour concentrated supernatant - 679 counts min^{-1}.
G : 2 hour concentrated supernatant - 678 counts min^{-1}.
H : 4 hour concentrated supernatant - 678 counts min^{-1}.
I : 6 hour concentrated supernatant - 679 counts min^{-1}.

Tracks K to T are autoradiographs of the corresponding tracks A to J showing ^{35}S-methionine labelled proteins in the sample.

CHAPTER FOUR

4. Development of an assay system to monitor VT gene expression

4.1 Introduction

The project was undertaken to examine the influence of various nutritional and physical parameters on toxin expression. A rapid simple and specific test to detect and quantify the amount of toxin produced was therefore required. At the time three types of assay to identify VTEC and their toxins were available (section 1.8) :-

(1) Cell culture toxicity assays (section 1.8.3) - Vero and HeLa cells are used in tissue culture to test for cytotoxicity and neutralisation of cytotoxicity with specific antibodies. Whilst these bioassays are highly sensitive, they are slow, expensive, labour intensive and reproducibility is difficult to achieve.

(2) Enzyme-linked immunosorbent assay (ELISA) methods, (section 1.8.4) - are more accurate, simpler, rapid and less expensive than the Vero/Hela cell assays but not as sensitive in that only high/moderate levels of VT are detected.

(3) Specific DNA probes (section 1.8.8) - are rapid, specific and sensitive but expensive. They give no indication as to the levels of VT produced (i.e. a cell may possess the appropriate gene sequence but it may only be expressed under certain conditions).

Two lines of research were pursued to develop an assay which would enable quantification of VT1 and VT2 gene expression at the molecular level :-

(1) Development of a VT RNA assay.

(2) Formation of a VT2-alkaline phosphatase gene fusion.

These are considered separately in sections 4.2 and 5.2 respectively.

4.2 Development of a RNA assay

One of the primary uses of RNA isolation procedures is the analysis of gene expression. In order to elucidate specifically VT1 and VT2 expression, an assay was developed that involved probing of Northern dot blots with DNA probes for VT1 and VT2 sequences. The basic protocol for this assay, termed the Verotoxin RNA assay, is shown in figure 4.1. The assay would enable the effects of various parameters on VT gene expression to be determined by measuring changes in the overall steady state level of *slt* operon transcription (i.e. VT messenger RNA (mRNA) synthesis). It is, however, assumed that changes in transcription of the genes reflect changes in the rate of transcription and hence changes in toxin production. Comparisons are made against a control set of conditions in each individual hybridisation with data for VT expression normalised so that the amount of mRNA in the control was 100%. As variations can occur in the degree of probe oligo-labelling, only relative changes and not direct comparisons between each hybridisation can be made. The relative expression can then be calculated by the formula :-

$100 \times (VT_{sample}/VT_{control})$.

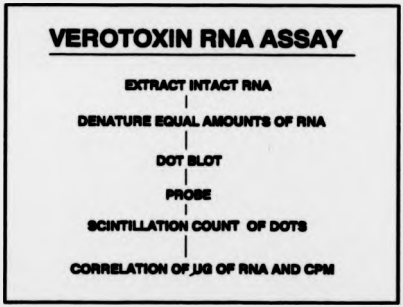
To verify mRNA recovery, blots could subsequently be stripped by boiling in water and then reprobed with a DNA probe to an abundant *E. coli* protein.

4.2.1 Preparation and probing of RNA

Northern hybridisation can be used to accurately determine the steady state level of a particular mRNA and initial experiments focussed on extracting intact RNA to give discrete bands on a Northern blot. Most procedures for isolating RNA from a biological source involve disruption of the cells, followed by steps to remove DNA and protein. A method for extracting RNA was adopted which was small scale and reproducible, enabling rapid sampling over a time course (section 2.30). Cells were

lysed in the presence of SDS and salt to denature the protein and precipitate the chromosomal DNA. After protein removal by organic extraction, the remaining RNA was recovered and concentrated by ethanol precipitation. Although deproteinisation should have prevented potential degradation of the cellular RNA by bacterial ribonuclease (RNase), rigorous precautions were taken to prevent incidental contamination by RNases from other sources (section 2.30). Any water used in RNA preparation was treated with diethylpyrocarbonate (DEPC). This inactivates RNases by covalent modification. However, solutions containing Tris cannot be effectively treated with DEPC since Tris contains a primary amine that reacts with DEPC causing inactivation.

Figure 4.1 **Protocol for the Verotoxin RNA assay**



The assay involves extracting intact RNA to give discrete bands on a Northern blot. Northern dot blots, adjusted to contain the same amount of RNA, are transferred to nitrocellulose using a S & S manifold dot blot. After baking, prehybridisation, hybridisation and washing, dots are cut out, placed in a scintillation vial and counted. A quantitative relationship between μg RNA and counts per minute can then be made.

Typically (unless otherwise stated), the RNA concentration was determined and samples, adjusted to contain the same amount of total RNA (as stated), were denatured in formaldehyde loading buffer (FLB) and electrophoresed on a formaldehyde denaturing gel (sections 2.31 & 2.32). Either formaldehyde or glyoxal/DMSO can potentially be used as the denaturant. However, formaldehyde gels were used in this study as they do not smear as much as glyoxal gels and can be photographed immediately using FLB. Also, agarose/formaldehyde gels allow good electrophoretic separation of RNAs over a wide range of molecular weights (MW), (Selden, 1989). RNA MW markers (BRL), VT1 and VT2 control DNA probe fragments were also denatured and electrophoresed (unless stated otherwise). The separated RNA was then transferred to a nitrocellulose membrane as described in section 2.33. Essentially transfer was exactly as for Southern blotting (section 2.18), except that the gel was pretreated with salt and not alkali since treatment of the gel with alkali and neutralisation buffer had been found to substantially reduce the efficiency of transfer of RNA from the gel to the nitrocellulose paper, particularly for larger RNAs (Thomas, 1980). Northern filters were probed exactly as for Southern filters, except that washes were less stringent (section 2.33(a)).

4.2.2 Northern blotting of VT-producing *E. coli*

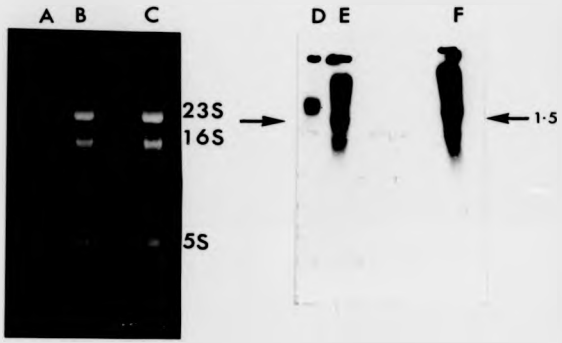
E. coli O157:H7 (ATCC 35150) contains both *slt*-I and *slt*-II coding sequences (section 3.5.2). Studies have shown that the *slt*-I and *slt*-II operons are both transcribed from polycistronic mRNA from a promoter upstream of the *slt*A gene (section 1.4.2). The size of the polycistronic mRNA species produced from each operon was estimated from published *slt*-I and *slt*-II sequence data (Calderwood *et al.*, 1987; Jackson *et al.*, 1987b respectively) to be approximately 1.6 Kb (for *slt*-I) and 1.7 Kb (for *slt*-II).

Figure 4.2 (tracks B & C) shows electrophoresed total RNA prepared from a stationary

Figure 4.2 **Northern blot analysis of *E. coli* O157:H7 (ATCC 35150)**

Total cellular RNA was denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 80V. The RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA fragments as described in materials and methods.

Key to tracks
A: RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
B: Total RNA isolated from *E. coli* O157:H7 (ATCC 35150) - concentration unknown.
C: Total RNA isolated from *E. coli* O157:H7 (ATCC 35150) - concentration unknown.



Tracks D & E correspond to tracks A and B respectively probed with a ^{32}PdCTP labelled 0.75 Kb *Hinc*II fragment from plasmid NTP705 which contains VT1 B subunit coding sequences. Track F corresponds to track C probed with a ^{32}PdCTP labelled 0.85 Kb *Sma*I-*Pst*I fragment from plasmid NTP707 which contains VT2 A subunit coding sequences. Filters were washed in 1 x SSC at 65^{o}C for 1 hour and exposed to autoradiographic film for 21 days at -70^{o}C.

phase culture of *E. coli* O157:H7 (ATCC 35150) and the subsequent blots obtained after probing Northern filters with the ^{32}P-dCTP labelled VT1 and VT2 probes (tracks E & F respectively). RNA was prepared as described in section 3.30 except that in this case attempts were made to extract the RNA from a 50 ml cell pellet. The cell concentration was, however, too high and a small aliquot of this was removed for RNA preparation. It can be seen that upon electrophoresis of 5 μl of the O157:H7 (ATCC 35150) cellular RNA, the 23S, 16S and 5S ribosomal RNA (rRNA) bands are clearly visible, indicating that there has been no gross degradation of the RNA. The corresponding Northern blots, after washing with 2 x SSC, 0.1 % (w/v) SDS and then 1 x SSC, 0.1% (w/v) SDS at 65^{o}C for 1 hour, showed evidence of hybridisation to the labelled DNA probes (tracks E & F). Non-specific hybridisation with material within the RNA marker lane was also observed (track D). There was no apparent difference in the pattern of the hybridised material in tracks E & F. The arrowed fragments, estimated to be approximately 1.5 Kb, were thought to correspond to the polycistronic mRNA produced by transcription of the *slt* operons in this strain. In an attempt to wash away the non-specifically bound material, filters were washed more stringently with 0.1 x SSC, 0.1% (w/v) SDS at 65^{o}C for 1 hour. After 9 days exposure to autoradiographic film at -70^{o}C, however, the filters showed no evidence of hybridisation. This could have been a consequence of :-

(a) The filters were over 4 weeks old and it is possible that any ^{32}P-labelled material had decayed to such an extent as to be undetectable, or, (b) the labelled probes had been removed by the stringent washing conditions.

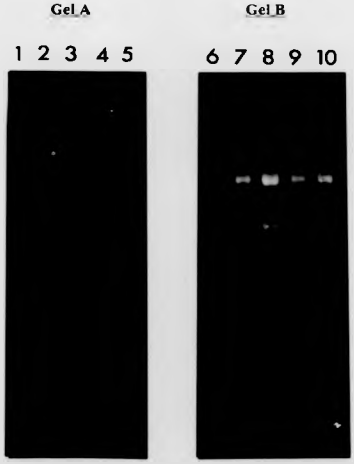
Figure 4.3 shows electrophoresed total RNA prepared from 1.5 ml overnight cultures of VT producing strains E3787, E32511, O157:H7 (ATCC 35150) and O157:H7 (CI), (section 2.30). Gel B contains double the amount of RNA per track (i.e. 10 μl per track) as gel A. Northern filters were washed in either 2 x SSC, 0.1% (w/v) SDS, at 65^{o}C for 45 min (gel A) or 2 x SSC, 0.1% (w/v) SDS, at room temperature for 45 min (gel B). Other than the DNA fragment controls, autoradiographs showed no evidence

Figure 4.3 Northern blot analysis of VT-producing *E. coli*

Duplicate samples of total cellular RNA from the strains indicated were denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 80V (gel A) and 30V (gelB). Gel B contained double the amount of RNA as gel A (concentration unknown). RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA fragments as described in materials and methods. Only one half of each gel is illustrated.

Key to tracks (Gels A & B)
1 & 6 : RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
2 & 7 : Total RNA isolated from *E. coli* E3787.
3 & 8 : Total RNA isolated from *E. coli* E32511.
4 & 9 : Total RNA isolated from *E. coli* O157:H7 (ATCC 35150).
5 & 10 : Total RNA isolated from *E. coli* O157:H7 (CI).

8 μl of VT1 *Hinc*II DNA probe fragment and VT2 *Sma*I-*Pst*I DNA probe fragment were included on each gel as controls.



of hybridisation with either probe after exposure to the Northern filters for 4 days and 27 days at -70°C (autoradiographs not shown). DNA control probe fragments hybridised specifically with the homologous probes as a dark smear from the well of the track due to the denaturing conditions employed.

In another experiment RNA was prepared from *E. coli* strains 60R363 and 60R746 from which the VT1 and VT2 probes had been prepared (figure 4.4). Upon probing of Northern filters with the labelled VT1 and VT2 probes and washes in 2 x SSC, 0.1% (w/v) SDS and 1 x SSC, 0.1% (w/v) SDS at room temperature for 1 hour each, the control probe fragments were seen to hybridise with the homologous probes as expected. In addition, bands in the wells of tracks 2 & 6 hybridised with the VT1 probe and the VT2 probe respectively. This was thought to be due to plasmid DNA contamination and to confirm this, RNA samples were treated with deoxyribonuclease (DNase) as described in section 2.34. 20 μl of the DNase treated RNA was denatured and electrophoresed as previously described. Subsequent autoradiographs only showed hybridisation to the control DNA probe fragments, confirming that contamination with plasmid DNA had occurred in figure 4.4 (autoradiographs not shown).

4.2.3 Probing of RNA from different points in the growth cycle of VT-producing strains

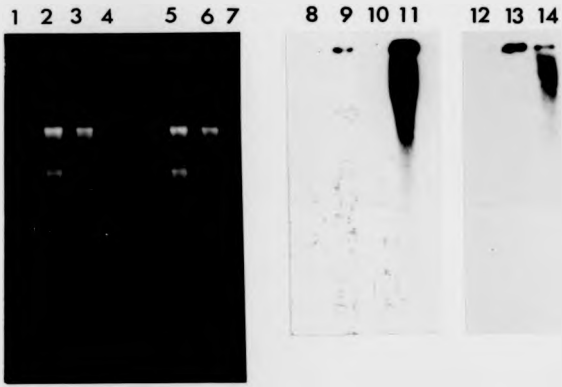
It was not known whether VT mRNA synthesis occurred throughout growth of the organisms or only at specific points in the growth cycle. In an attempt to detect VT mRNA transcripts, RNA was prepared from *E. coli* strains E3787, E32511, 60R746 and 60R363 at 2 (exponential), 4 (early stationary) and 6 (stationary) hours culture as described in section 2.36. Samples, adjusted to contain 15 μg of cellular RNA were denatured, electrophoresed on formaldehyde gels and Northern blotted as described above (figure 4.5). Filters were washed in 2 x SSC, 0.1% (w/v) SDS and 1 x SSC, 0.1% (w/v) SDS at room temperature for 1 hour each. After three weeks exposure at -70°C,

Figure 4.4 Northern blot analysis of *E. coli* strains 60R746 & 60R363

Total cellular RNA was denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 60V. The RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA fragments as described in materials and methods.

Key to tracks

1 : RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
2 & 5 : 5 μl of total RNA isolated from *E. coli* 60R746.
3 & 6 : 5 μl total RNA isolated from *E. coli* 60R363.
4 : 8 μl 0.75 kb *Hinc*II VT1 DNA probe fragment.
7 : 8 μl 0.85 kb *Sma*I-*Pst*I VT2 DNA probe fragment.



Tracks 8-11 and 12-14 correspond to tracks 1-4 and 5-7 after probing with a ^{32}PdCTP labelled 0.75 Kb *Hinc*II fragment of plasmid NTP705 which contains VT1 B subunit coding sequences and a ^{32}PdCTP labelled 0.85 Kb *Sma*I-*Pst*I fragment of plasmid NTP707 which contains VT2 A subunit coding sequences. Filters were washed in 1 x SSC at room temperature and exposed to autoradiographic film for 3 days at -70^{0}C.

Figure 4.5 Northern blot analysis of RNA isolated from different points in the growth phase of *E. coli* 6OR746 and 6OR363

Total cellular RNA was prepared from *E. coli* strains 6OR746 and 6OR363 at the times shown. Duplicate samples, adjusted to contain 15 μg of RNA, were denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 80V. RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA probes as described in materials and methods.

Key to tracks
A : RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
B-D : Total RNA isolated from 6OR746 at 2, 4 and 6 hours growth respectively.
E-G : Total RNA isolated from 6OR363 at 2, 4 and 6 hours growth respectively.

autoradiographs showed no evidence of hybridisation except to the control probe fragments (results not shown).

4.2.4 Determination of VT messenger RNA half-life

An important characteristic of prokaryotic mRNA is that its half-life is short compared to other types of prokaryotic RNA molecules. For bacteria, the half-life of a typical mRNA molecule is a few minutes. This feature has an important regulatory function. If a protein is no longer required, a cell need only turn off synthesis of the mRNA that encodes the protein, soon afterwards, none of that particular mRNA will remain and synthesis of the protein will no longer occur. One possible explanation for the inability to detect VT mRNA was that the transcripts once produced were being degraded very rapidly by the cell. In order to determine the mRNA half-lives for VT1 and VT2 (section 2.35), RNA was isolated from late exponentially growing cultures of VT-producing strains E3787 and E32511 at known times (in minutes), after the addition of 100 μg ml^{-1} of rifampicin, an inhibitor which specifically blocks RNA synthesis by interaction with the DNA-dependent RNA polymerase. Rifampicin inhibits chain initiation by binding to the β subunit of RNA polymerase thereby blocking the transition from the chain initiation to the elongation phase. Rifampicin and related drugs have been used extensively in investigations of transcription as they can be added to a growing culture for *in vivo* studies or to a reaction mixture for *in vitro* studies and will rapidly inhibit the initiation of new chains. Since it takes only a few seconds to complete a growing RNA chain, RNA synthesis is quickly terminated. Samples, adjusted to contain 5 μg of RNA, were denatured in FLB and electrophoresed overnight on formaldehyde gels.

In both *E. coli* strains it appeared that there was little change in the amount of total RNA present in the cell population until thirty minutes after addition of rifampicin (figure 4.6). After this time there appeared to be gross turnover in the total RNA with

Figure 4.6 Northern blot analysis of RNA isolated from *E. coli* E3787 and E32511 after addition of rifampicin

Total cellular RNA was prepared at the times shown after addition of rifampicin to a final concentration of 100 $\mu g\ ml^{-1}$. Duplicate samples adjusted to contain 5 μg of RNA were denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 30V overnight. The RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA fragments as described in materials and methods.

Key to tracks
A & J: RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
B to I: Total RNA isolated from *E. coli* E3787 after 0, 5, 10, 20, 30, 40, 50 & 60 minutes respectively.
K to Q: Total RNA isolated from *E. coli* E32511 after 0, 5, 10, 20, 30, 40 & 60 minutes respectively.

Tracks R to Z correspond to tracks A to I after probing with a ^{32}PdCTP labelled 0.75 Kb *Hinc*II fragment from plasmid NTP705 which contains VT1 B subunit coding sequences. The filter was washed in 1 x SSC at room temperature and exposed to autoradiographic film for 5 days at -70^{o}C.

visible deterioration of the rRNA subunits (track F & O). After probing, Northern filters showed no evidence of hybridisation except for a band of less than 0.24 Kb in tracks H & I which hybridised with the VT1 *Hinc*II fragment (tracks Y & Z). This band is much smaller than the expected size of a transcript proceeding from the putative *slt*-I promoter to the terminator (Jackson *et al.*, 1987a) and most likely represents non-specific hybridisation to a degradation product. When the experiment was repeated, no hybridisation to the Northern filters was observed (results not shown).

4.2.5 Northern blot analysis of varying concentrations of cellular RNA

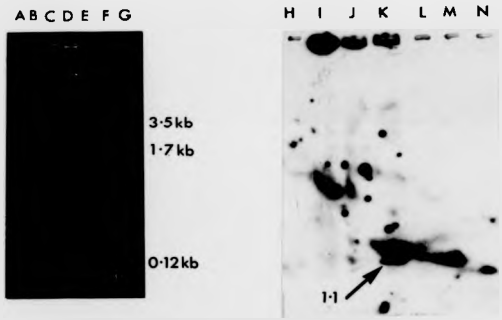
It was thought that the inability to detect the VT transcripts might have been due to insufficient loading of RNA on the formaldehyde gels. To prepare a much more concentrated RNA sample, RNA was extracted from late exponential growing cultures of *E. coli* strains 60R363 and 60R746 as described in section 2.30, except that 15 ml of culture was pelleted for extraction and the final RNA precipitate was resuspended in 100 μl of SAE. Samples, adjusted to contain 50, 100, and 200 μg of cellular RNA from each strain, were electrophoresed and transferred to nitrocellulose as previously described.

The resulting agarose gel is shown in figure 4.7. The RNA markers appeared to have degraded, however, the rRNA subunits can be used as internal size standards as they correspond to molecular sizes of 3.5, 1.7 & 0.12 Kb for the 23S, 16S and 5S subunits respectively (Sung *et al.*, 1990). Duplicate filters were probed under two different protocols, as described in sections 2.33(a) & 2.33(b). No hybridisation was observed except with a fragment of approximately 1.1 Kb (track K), with the VT1 labelled probe using protocol 2.33(a). As previously mentioned, strain 60R746 is an *E. coli* K12 strain carrying the recombinant plasmid NTP705. Plasmid NTP705 carries a 0.75 Kb *Hinc*II fragment containing sequences encoding the B subunit of VT1 (Willshaw *et al.*, 1987) cloned into the ampicillin resistance (Ap^r) gene of the plasmid cloning vector

Figure 4.7 Northern blot analysis of RNA isolated from *E. coli* strains 60R746 and 60R363

Duplicate samples of total cellular RNA prepared from late exponential growing cultures of *E. coli* strains 60R746 and 60R363 were adjusted to contain the amounts of RNA indicated, denatured in formaldehyde loading buffer and electrophoresed on a 1.5% (w/v) agarose, 2.2 M formaldehyde gel in 1 X MOPS buffer at 30V overnight. RNA was transferred to nitrocellulose and probed with ^{32}PdCTP labelled VT1 and VT2 DNA probes as described in materials and methods.

Key to tracks
A : RNA markers (BRL). Fragment sizes were 9.49, 7.46, 4.4, 2.37, 1.4 & 0.24 Kb.
B : Total RNA isolated from *E. coli* 60R746 (50 μg).
C : Total RNA isolated from *E. coli* 60R746 (100 μg).
D : Total RNA isolated from *E. coli* 60R746 (200 μg).
E : Total RNA isolated from *E. coli* 60R363 (50 μg).
F : Total RNA isolated from *E. coli* 60R363 (100 μg).
G : Total RNA isolated from *E. coli* 60R363 (200 μg).



Tracks H-N correspond to tracks A-G after probing with a ^{32}PdCTP labelled 0.75 Kb *Hinc*II fragment from plasmid NTP705 which encodes VT1 B subunit sequences. The filter was washed in 1 x SSC at room temperature and exposed to autoradiographic film overnight at -70^{0}C.

pACYC177 (Willshaw *et al.*, 1985). Therefore, the 1.1 Kb fragment cannot correspond to polycistronic mRNA transcribed from the promoter upstream of the A subunit gene of *slt*-I. There are two possible explanations for the presence of a mRNA transcript containing *slt*-IB coding sequence. Firstly, transcription from the RNA polymerase recognition site for the Ap^r gene in which the 0.75 Kb *Hinc*II fragment is cloned is being initiated. Transcriptional readthrough of the *slt*-IB subunit sequence occurs, with termination of mRNA transcription from either the termination sequence of the Ap^r gene or from the termination sequence encoded in the cloned B subunit gene of VT1. Alternatively, transcription of the *slt*-IB coding sequence could occur independently from a promoter upstream of the *slt*-IB gene. Although there has been no direct evidence for a second promoter for independent transcription of the *slt*-IB gene, existance of the latter has not been precluded (De Grandis *et al.*, 1987; Newland *et al.*, 1985; Weinstein *et al.*, 1988b). Furthermore, an examination of the transcription of the Shiga toxin operon (*stx*) by Northern blot analysis has revealed both a polycistronic message (about 1.7 Kb in size) and a putative B subunit message (about 0.7 Kb), (Koslov *et al.*, 1988). The 1.1 Kb fragment however, is too large to represent a mRNA species produced by transcription from an independent promoter for the *slt*-IB gene, unless termination of transcription occurs at a point downstream of the *slt*-IB RNA polymerase termination site, perhaps from the termination sequence of the Ap^r gene. To explore these hypotheses, a detailed study of the insertion of the 0.75 Kb *Hinc*II fragment within the Ap^r gene of pACYC177 would be required. This, however, was not pursued during the course of this project.

Whatever the explanation, it is obvious that either the mRNA transcript containing the *slt*-IB coding sequence is not highly expressed (with hybridisation only being observed when 200 μg of RNA was loaded), or the sensitivity of the Northern analysis is insufficient to enable detection of the expressed mRNA transcript at lower total RNA concentrations.

4.3 Summary and overview

In the development of an RNA assay system to specifically quantify VT1 and VT2 expression, experiments were performed to extract intact RNA from VT-producing strains for Northern blotting. Although RNA extraction and electrophoresis proved successful, subsequent probing of Northern filters to produce discrete bands on a Northern blot was not achieved.

The most likely cause of problems in a Northern analysis is poor quality of the RNA being analysed. The major source of failure in any attempt to produce RNA is contamination by RNase. RNases are very stable, active enzymes that require no cofactors to function, therefore, a small amount of RNase in an RNA preparation will create a real problem. However, great care was taken to avoid RNase contamination and rRNA bands remained sharp, indicating there was no gross degradation and, hence, no substantial contamination with RNase during sample preparation.

Both denatured and native RNA molecules are capable of binding to nitrocellulose filters. In order to transfer efficiently from agarose gels, however, the RNA must be denatured (Thomas, 1980). It is possible that RNA transfer may not have been successful, the conditions may not have been appropriate or degradation of the message could have occurred upon RNA transfer to the nitrocellulose and subsequent probing. Presoaking of the gel in high salt and staining the gel with ethidium bromide has been found to reduce transfer of eukaryotic RNA to nitrocellulose (Thomas, 1980). When DNA control probe fragments were run on gels, hybridisation with the VT DNA probes was observed, however, nitrocellulose has a higher binding capacity for DNA compared to RNA. To confirm successful transfer of RNA the Northern filters could have additionally been probed with a DNA probe to an abundant *E. coli* protein.

The most obvious reason for the inability to detect transcripts was lack of sensitivity due to the toxin genes not being expressed sufficiently under the conditions employed to enable detection of the mRNA with the VT1 and VT2 DNA probes. Selden (1989) recommends that 0.5 to 10 μg of RNA be probed, however, Sung *et al.* (1990) subsequently published data in which they showed detection of the *slt*-II mRNA transcript. In the latter case, 50 μg of total cellular RNA was electrophoresed and probed with, on average, 5-10 times more RNA than probed in the present study. Also the RNA was prepared from an *E. coli* strain carrying the entire *slt*-II operon cloned into a high copy number plasmid vector pBR328, whereas in the present study RNA was typically prepared from VT-producing strains in which the VT operons were chromosomally encoded. In the study of regulation of gene expression it is desirable to have a single copy of the operon of interest as the presence of cloned genes on multicopy plasmids can cause misleading or erroneous results that do not accurately reflect the "normal" level of transcription. This is largely due to the fact that plasmid copy number can vary with growth conditions, the size of DNA insert, as well as the presence of other plasmids (Adams & Hatfield, 1984).

Further development of this assay protocol was suspended due to the difficulties encountered. If research in this area had been continued, attempts would have been made to increase expression of the VT operons by using mitomycin C (section 1.4.4.5), and growth under low iron conditions (section 1.4.4.3).

CHAPTER FIVE

5. Formation of a *slt*-II::Tn*phoA* gene fusion

5.1 Introduction

Protein fusions have played a central role in molecular genetic studies of the mechanism of protein export in bacteria (Michaelis & Beckwith, 1982; Beckwith & Silhavy, 1983). Conventionally this approach involves the fusion of a selectable "reporter" gene, which possesses an easily assayed activity, to the promoter and controlling regions of the particular gene under study. The "reporter" gene may be used in either transcriptional (operon) fusions, where it retains its own translational start but is dependent on the attached DNA for transcription, or in translational protein fusions, where both transcription and translation are dependent on signals in the attached upstream DNA.

Most studies have utilised hybrids containing amino-terminal sequences of an exported protein fused to the *lacZ* gene that encodes the cytoplasmic protein β-galactosidase. Use of such hybrid proteins is limited however, as they are frequently lethal to the cell when produced in large amounts because of the inability of the β-galactosidase moiety to pass through the bacterial cytoplasmic membrane (Bassford *et al.*, 1979). This phenotype is known as "over-production lethality" where the hybrid protein is thought to contain sequences that block the cell export machinery, thus hindering normal protein export.

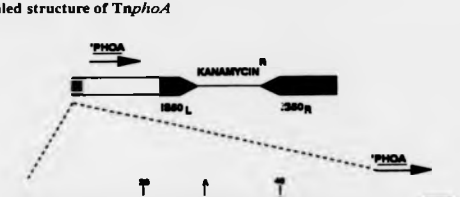
Subsequently, Hoffman & Wright (1985) constructed a set of plasmids which encode the gene (*phoA*) for the *E. coli* periplasmic protein alkaline phosphatase (PhoA), missing its own signal sequence. Enough of the mature protein is retained such that highly active PhoA can be generated *in vitro* by attachment to *phoA* of appropriate restriction fragments containing the amino termini of other proteins. PhoA only displays activity when exported from the cytoplasm as it must dimerise to be active and the environment

in the cytoplasm of the bacterial cell is too reducing to allow the formation of the necessary disulphide bonds. Manoil & Beckwith (1985) extended this approach with the formation of Tn*phoA*, a transposon vector that allows generation of gene fusions between the amino terminal portion of a target gene and the coding sequence of bacterial PhoA *in vivo*. A derivative of Tn5, Tn*phoA* is composed of *phoA* lacking its promoter, its translation initiation site, and the DNA corresponding to the signal sequence and first five amino acids of the protein (figure 5.1 (a)). Hybrid proteins expressing PhoA activity only occur when Tn*phoA* inserts in the proper orientation and reading frame to a signal that promotes the export of the protein from the cytoplasm. This signal can correspond to those found in periplasmic, outer membrane, cytoplasmic membrane or secreted proteins. As most bacterial virulence determinants are expressed at the cell surface, this technique selects for Tn*phoA* insertions into such genes. Hybrid proteins generated by Tn*phoA* insertion contain 17 amino acid residues at their fusion joints resulting from the translation of Tn5 and linker sequences. This junction sequence neither blocks export nor acts as an export signal for the hybrid proteins (Manoil & Beckwith, 1985). The use of transposon Tn*phoA* combines the advantages of working with hybrid proteins able to be secreted (Hoffman & Wright, 1985), with the versatility of Tn5 transposition in generating hybrids (Bruijn & Lupski, 1984). PhoA is an easily assayable enzyme which can be detected even at low levels in bacterial colonies by the use of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (XP). When PhoA hydrolyses XP in an appropriate agar medium *E. coli* colonies are blue.

The use of *phoA* fusions has proven to be a powerful tool for the study of protein secretion, membrane protein topological structure, protein export signals and the identification of genes for cell envelope and extracellularly secreted proteins (for a review, see Manoil *et al.*, 1990). Use of the sensitive indicator media and enzyme assay for PhoA (section 2.44.2), also provides a simple way to monitor the expression of the hybrid gene, which in turn reflects the activity of the exogenous promoter. There are

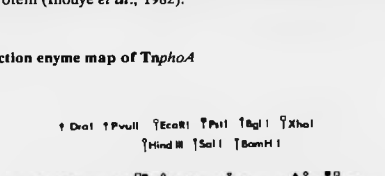
Figure 5.1 **The transposon vector Tn*phoA*** (Manoil & Beckwith, 1985)

(a) **Detailed structure of Tn*phoA***



There are 50 base pairs (bp) at the beginning of the *phoA* coding region. Of this sequence, 48 bp are derived from IS50L of transposon Tn5 and 2 bp originate from the *Pst*I linker of plasmid pCH39 (Hoffman & Wright, 1985). The amino acid residues that are shown are present at the fusion joint of every hybrid protein generated. The DNA sequence differs from that of the IS50L region of Tn5 by an A-to-G change at position 29. All of the *phoA* is encoded except the signal sequence and 5 amino acid residues of the mature protein (Inouye *et al.*, 1982).

(b) **Restriction enyme map of Tn*phoA***



significant advantages to using such an approach. Whereas *lac* operon fusions limit analysis of regulation to the transcriptional level, *phoA* gene fusions allows screening for an effect of a given regulatory parameter at any step in gene expression, including transcription, mRNA processing or stability, and translational or post-translational controls.

Gene fusion technology has previously been exploited to assess the regulation of VT1 expression by iron (Calderwood & Mekalanos, 1987). This involved the creation of hybrid genes between the promoter and proximal portions of *slt*-IA and Tn*phoA* (figure 5.2). The VT1::Tn*phoA* gene fusion plasmid, pSC105 was kindly provided by Dr S Calderwood, Harvard University, to enable study of VT1 gene expression. In order to quantitate VT2 gene expression, fusions between Tn*phoA* and the cloned *slt*-II operon carried on plasmid NTP707 were made in this study. The protocol for formation of these fusions is outlined in figure 5.3 and discussed in detail in section 5.2.

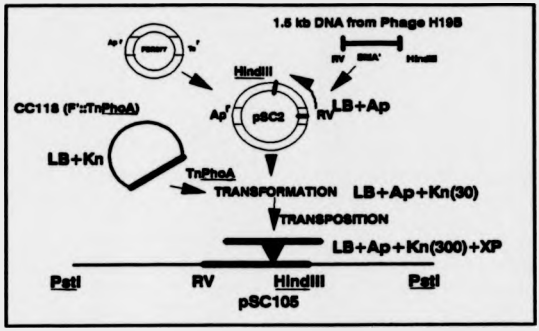
In the formation of a *slt*::Tn*phoA* gene fusion (figure 5.4), both the A and B subunits are synthesised with amino terminal signal sequences. Tn*phoA* can therefore fuse into either subunit gene and be exported into the periplasm to give enzyme activity. As both genes are transcribed from a single promoter upstream of the A subunit gene in *slt*-II both possible gene fusions allow monitoring of VT2 gene expression to be undertaken.

5.2 Results

5.2.1 Isolation of *slt*-II::Tn*phoA* gene fusions

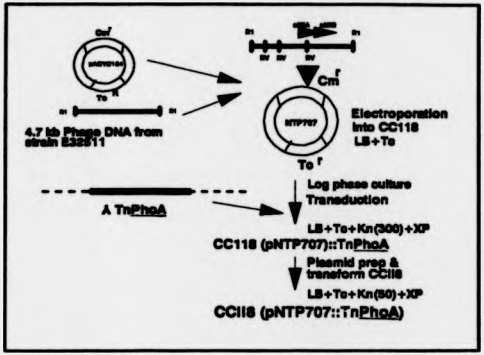
Plasmid NTP707 contains a 4.7 Kb *Eco*RI fragment encoding the entire *slt*-II operon, inserted into the chloramphenicol resistance (Cm^r) gene of plasmid pACYC184 (table 3.2). *E. coli* CC118 (table 2.1), which is PhoA negative, was electroporated with caesium chloride density gradient purified pNTP707 (section 2.37.1) and the

Figure 5.2 **Schematic diagram of the protocol for the formation of plasmid pSC105**



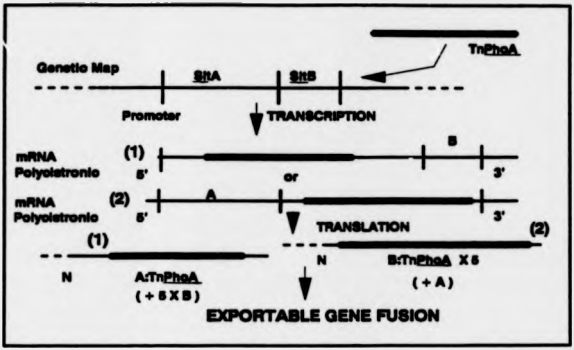
Calderwood & Mekalanos (1987) cloned a 1.5 Kb *Eco*RV/*Hind*III fragment from phage H19B into plasmid pBR377. This fragment contained the promoter and proximal portion of the *slt*-IA subunit gene. The recombinant plasmid pSC2 was transformed into *E. coli* CC118, carrying Tn*phoA* on a F' plasmid. Growth of transformants on plates containing Kn (300 μg ml^{-1}) selected for transposition of Tn*phoA* onto the high copy number plasmid vector. CC118 was retransformed with plasmid preparations of the transformants and blue colonies on LB+Ap+Kn(30)+XP plates isolated. These contained in-frame fusions of Tn*phoA* to secreted gene products. The presence and location of Tn*phoA* within pSC105 was subsequently confirmed by restriction mapping.

Figure 5.3 **Schematic diagram of the protocol for the formation of a *slt*-II::Tn*phoA* gene fusion**



The recombinant plasmid NTP707 (Willshaw *et al.*, 1987) was electroporated into *E. coli* CC118 and transformants selected on LB+Tc plates. Tn*phoA* was introduced by transduction of a log phase culture of CC118 (pNTP707) with lambda Tn*phoA* (Gutierrez *et al.*, 1987). Growth of transformants on LB+Tc+Kn(300)+XP plates selected for transposition of Tn*phoA* onto the high copy number plasmid vector. CC118 was retransformed with plasmid preparations of the transformants and blue colonies on LB+Tc+Kn(50)+XP plates isolated. These contained in-frame fusions of Tn*phoA* to secreted gene products on plasmid NTP707.

Figure 5.4 **Schematic diagram to illustrate the potential gene fusion products from random insertion of Tn*phoA* into the *slt* operon**



The structural genes for A and B polypeptides of VT, *slt*-A and *slt*-B respectively, are organised into an operon (*slt*) which is transcribed from a promoter upstream of the A subunit gene to produce a polycistronic messenger RNA (Sung *et al.*, 1990). As the A and B subunit genes both possess amino terminal signal sequences, insertion of Tn*phoA* downstream of the signal peptidase cleavage site in either subunit gene will result in an exportable gene fusion product.

transformants plated onto LB plates containing tetracycline (Tc) to select for pNTP707. Plasmid DNA was prepared from transformants displaying Tc resistance (Tc^r) as described in section 2.13.2, restricted with *SmaI-PstI* and electrophoresed on an agarose gel. Restriction with *SmaI-PstI* produced the expected 8 and 0.85 Kb fragments (figure 5.5), confirming the presence of pNTP707 within *E. coli* CC118.

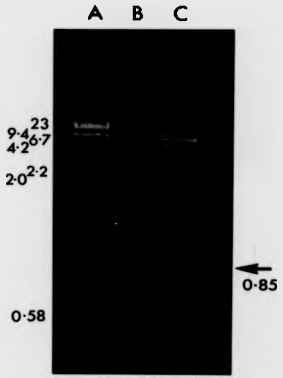
Specially designed broad host-range plasmid vectors carrying Tn*phoA* have predominantly been used to deliver Tn*phoA* into target genes of a variety of Gram-negative bacteria (Taylor *et al.*, 1987). Typically the broad host-range plasmid vector is introduced into the target organism by conjugation, whereupon transposition of the plasmid encoded Tn*phoA*, which encodes kanamycin resistance (Kn^r), into the chromosome is caused by superinfection with an incompatible plasmid and selection for Kn^r (Taylor *et al.*, 1987; Peterson *et al.*, 1988; Donnenberg *et al.*, 1990). In the present study, Tn*phoA* was introduced into *E. coli* CC118 (pNTP707) on a defective lambda suicide phage as described in section 2.38. Lambda Tn*phoA* contains a nonsense mutation within a gene essential for bacteriophage replication (Gutierrez *et al.*, 1987). Such a bacteriophage can propagate in a suppressing (Sup^+) host, but is unable to establish itself in a non-supressing (Sup^0) host. The high concentration of Kn (300 μg ml^{-1}) preferentially selects for transposition of Tn*phoA* onto the high copy number plasmid vector (Berg *et al.*, 1983). Individual blue colonies on this medium after incubation at 37°C for 2 days, contained in-frame fusions of Tn*phoA* to secreted gene products, whereas white colonies represented those strains in which Tn*phoA* had not fused to an exported protein. To ensure Tn*phoA* was not chromosomally encoded, plasmid DNA was prepared from each blue fusion strain on a small scale (section 2.13.2) and used to transform freshly competent CC118 (section 2.37.2). Blue colonies on LB plates containing XP, Tc and Kn (30 μg ml^{-1}), contained in-frame fusions of Tn*phoA* to secreted gene products on NTP707. The presence and location of Tn*phoA* within nine fusion-bearing plasmids was then confirmed by restriction mapping. These

Figure 5.5 Agarose gel electrophoresis of plasmid DNA from CC118 (pNTP707)

Plasmid DNA prepared from *E. coli* CC118 (pNTP707) was restricted as indicated and electrophoresed on a 1% (w/v) agarose gel at 50mA in 1 x TBE and stained with ethidium bromide.

Key to tracks

A : Phage lambda DNA restricted with *Hind*III.
B : Plasmid DNA from CC118 (pNTP707) - unrestricted.
C : Plasmid DNA from CC118 (pNTP707) - *Sma*I/*Pst*I restricted.



were designated pSLF9, pSLF10, pSLF17, pSLF18, pSLF22, pSLF23, pSLF29, pSLF32 and pSLF34.

5.2.2 Restriction mapping of *slt*-II::Tn*phoA* fusions

Plasmids were prepared from the nine fusion bearing strains by small scale alkaline lysis (section 2.13.2), and digested with the enzyme *Eco*RV. This restriction endonuclease cuts plasmid NTP707 at one site in the vector plasmid pACYC184 (figure 3.2 (a)), and three sites in the 4.7 Kb *Eco*RI insert (figure 3.2 (b)). Transposon Tn*phoA* has no *Eco*RV restriction enzyme sites (figure 5.1 (b)). Thus *Eco*RV digestion of plasmid NTP707, without a Tn*phoA* insert, gives rise to four fragments of approximately 0.9, 1.3, 3.0, and 3.8 Kb (tracks 2 & 3, figure 5.6). It can be seen that in all the nine fusion bearing plasmids (tracks 4 to 12, figure 5.6), the 3.8 Kb *Eco*RV fragment carries the Tn*phoA* insert, leading to production of the expected 11.7 Kb large fragment. Comparison with the published nucleotide sequence of the *slt*-II operon cloned from *E. coli* strain 933 (figure 5.7), and the physical map of the *slt*-II operon (figure 5.8), shows that the *Eco*RV restriction enzyme site lies within the *slt-II* A subunit gene, 68 base pairs (bp) downstream from the N-terminal signal cleavage site of the VT2 A subunit. Thus in all nine fusion bearing plasmids, insertion of Tn*phoA* has occurred downstream of this *Eco*RV restriction site.

To more finely define the site of insertion of Tn*phoA* within these fusion plasmids, each plasmid was restriction mapped using single and multiple restriction endonucleases. The enzymes used were *Eco*RI, *Eco*RV, *Pst*I, *Sma*I, *Kpn*I/*Bam*HI and *Kpn*/*Sal*I. It became evident almost immediately from the pattern of *Eco*RI digested plasmid DNA (figure 5.9), that the location of the fusion joint was not randomly distributed over the whole *slt*-II operon. Clustering occurred at two different sites with pSLF17, pSLF18, pSLF22, pSLF23, pSLF29 and pSLF32 forming one group and pSLF9, pSLF10 and pSLF34 the other. Only restriction enzyme digests of a representative of each type of

Figure 5.6 **Agarose gel electrophoresis of the Tn*phoA*-insertion plasmids pSLF9, pSLF10, pSLF17, pSLF18, pSLF22, pSLF23, pSLF29, pSLF32 and pSLF34**

Plasmid DNA was restricted with *Eco*RV as indicated and electrophoresed on a 0.7% (w/v) agarose gel at 30V overnight in 1 x TBE and stained with ethidium bromide.

Key to tracks

1 : Phage lambda DNA restricted with *Hind*III.
2 : CsCl density gradient purified pNTP707 - *Eco*RV restricted.
3 : pNTP707 - *Eco*RV restricted.
4 to 12: pSLF9, pSLF10, pSLF17, pSLF18, pSLF22, pSLF23, pSLF29, pSLF32 & pSLF34 respectively - *Eco*RV restricted.

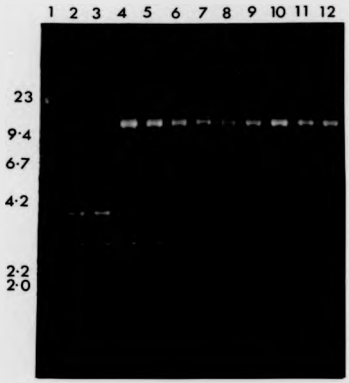
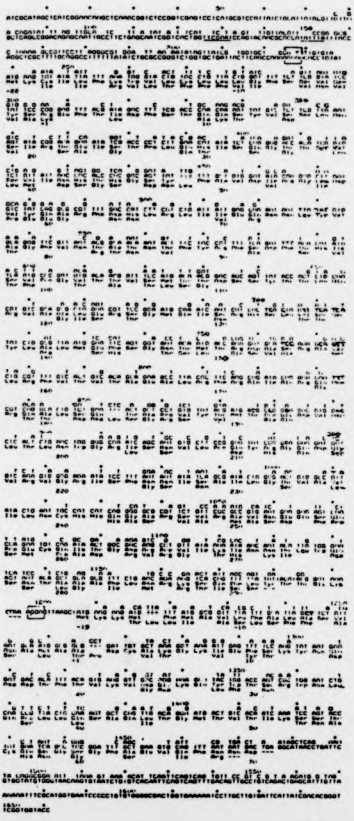


Figure 5.7 Nucleotide sequence of the *slt*-II operon cloned from *E. coli* strain 933 (Jackson *et al.*, 1987b)



The nucleotide sequence and deduced amino acid sequence is numbered above and below each line respectively. The open reading frame for the *slt*-IIA and *slt*-IIB subunits are between base pairs 239 & 1193 and 1207 & 1474 respectively with the N-terminal alanine in both cases depicted as +1. The proposed promoter sequences are underlined starting at bases 126 (-35 sequence) and 149 (-10 sequence). Two potential ribosome binding sites are boxed beginning at base pairs 225 and 1196 (for translation of *slt*-IIA & *slt*-IIB respectively).

Figure 5.8 **Restriction enzyme map of the slt-II operon** (Jackson *et al.*, 1987b)

Restriction sites are given within the 3250 bp *Sph*I to *Eco*RI fragment isolated from the hybrid plasmid pNN76. The location and orientation of the structural genes, *slt*-IIA and *slt*-IIB, are shown above the restriction map.

Figure 5.9 Agarose gel electrophoresis of *slt*II::Tn*phoA* gene fusion plasmids

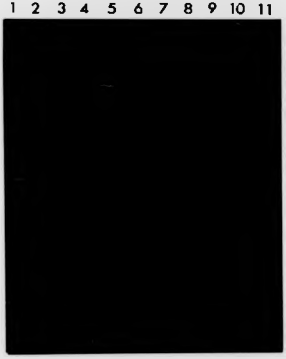
Plasmid DNA from the fusion plasmids indicated was restricted with *Eco*RI and electrophoresed on a 0.7% (w/v) agarose gel at 30V overnight in 1 x TBE and stained with ethidium bromide.

Key to tracks

1 : CsCl density gradient purified pNTP707 - *Eco*RI restricted.

2 : Alkaline lysis small-scale preparation of pNTP707 - *Eco*RI restricted.

3-11 : pSLF9, pSLF10, pSLF17, pSLF18, pSLF22, pSLF23, pSLF29, pSLF32 & pSLF34 respectively - *Eco*RI restricted.



insertion, pSLF22 and pSLF34, are shown for convenience (figures 5.10 & 5.11). From this data a restriction map of each type of insertion was determined as detailed in figure 5.12. All fusions mapped to the B subunit gene of the *slt*-II operon. Fusion plasmids pSLF22 (pSLF17, pSLF18, pSLF23, pSLF29 and pSLF32) mapped to a point approximately 100 bp downstream of the *Pst*I site, whilst pSLF34 (pSLF9 and pSLF10) mapped to a position approximately 200 bp further downstream.

As mentioned above, the high frequency at which the two types of fusion were isolated suggests that insertion of the transposon into the *slt*-II operon may not be random. Although Tn*phoA* has been reported to show a low sequence specificity of insertion (Manoil & Beckwith, 1985), such "hot-spot" insertion sites for Tn*phoA* have been described elsewhere (Gott & Boos, 1988). Alternatively the topological organisation of some fusion proteins might be lethal to the cell and therefore are not selected.

5.2.3 Sequence analysis of pSLF22 and pSLF34

As stated earlier, Tn*phoA* must insert itself in the correct orientation and reading frame to create an active fusion. Gott & Boos (1988), however, have shown Tn*phoA* to be capable of generating active fusions, even when inserted out of frame by +1. This is thought to be due to *in vivo* frameshifting where the translational machinery corrects the wrong frame by reading a sequence of four nucleotides as a sense codon.

To locate the exact site of insertion of Tn*phoA* and to determine whether in-frame fusions had been created, the dideoxynucleotide chain terminator/M13 vector method of DNA sequencing was initially applied to provide sequence data across the fusion joints of pSLF22 and pSLF34 (section 2.39.2). Large scale plasmid preparations of pSLF22 and pSLF34 were made (2.13.1(b)), and the appropriate *Pst*I/*Eco*RI fragment purified. Double digestion with *Pst*I and *Eco*RI was found to be inappropriate for isolation of the required *Pst*I/*Eco*RI fragments as comigration with internal *Pst*I

Figure 5.10 Restriction endonuclease mapping of pSLF22 and pSLF34

Plasmid DNA was restricted as indicated and electrophoresed on a 0.6% (w/v) agarose gel at 50mA in 1 x TBE and stained with ethidium bromide.

Key to tracks

1&11: Phage lambda DNA restricted with *Hind*III.
2-4: pNTP707, pSLF22 & pSLF34 respectively - *Eco*RI restricted.
5-7: pNTP707, pSLF22 & pSLF34 respectively - *Eco*RV restricted.
8-10: pNTP707, pSLF22 & pSLF34 respectively - *Pst*I restricted.

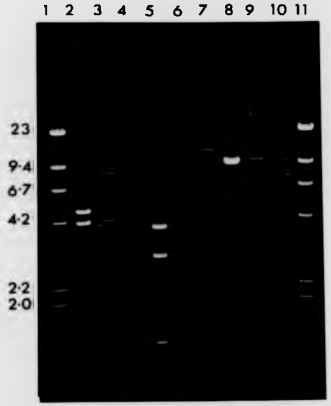


Figure 5.11 Restriction endonuclease mapping of pSLF22 and pSLF34

Plasmid DNA was restricted as indicated and electrophoresed on a 0.7% (w/v) agarose gel at 50mA in 1 x TBE and stained with ethidium bromide.

Key to tracks

1&6: Phage lambda DNA restricted with *Hind*III.

2 : pNTP707 - *Kpn*I restricted.

3-5: pNTP707, pSLF22 & pSLF34 respectively - *Kpn*I/*Sal*I restricted.

The additional fragments in tracks 4 & 5 that comigrate with the 6.78 Kb *Hind*III fragment in track 6 are due to partial digestion at the *Kpn*I site in pACYC184.

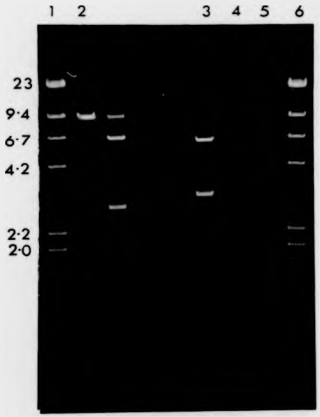
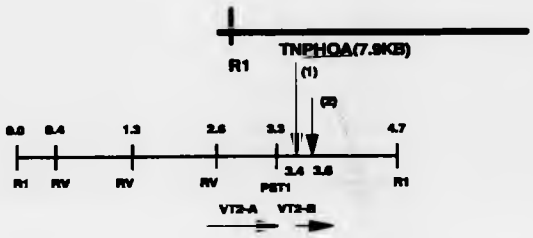


Figure 5.12 Partial restriction endonuclease map showing the relative positions of insertion of Tn*phoA* in fusion plasmids pSLF22 and pSLF34

Fusion joints between *slt*II and Tn*phoA* were estimated from restriction endonuclease mapping of the fusion plasmids pSLF22 and pSLF34.

Position (1) = pSLF22 (fusion plasmids pSLF17, pSLF18, pSLF23, pSLF29 & pSLF32 also mapped to this point).
Position (2) = pSLF34 (fusion plasmids pSLF9 & pSLF10 also mapped to this point).



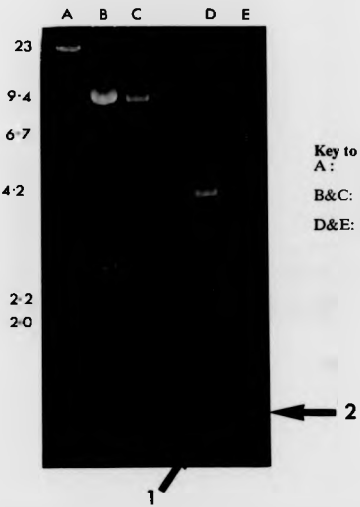
fragments of Tn*phoA* occurred in each case (figure 5.13). From figure 5.13 the sites of insertion of Tn*phoA* within each plasmid was more finely mapped to approximately 150 bp and 280 bp downstream of the *Pst*I site in pSLF22 and pSLF34 respectively. It was found that isolation of the appropriate *Eco*RI fragment, followed by *Pst*I digestion resulted in a larger yield of the required *Pst*I/*Eco*RI fragment than secondary *Eco*RI restriction of the isolated *Pst*I fragment. This was due to the production of partially digested fragments in the latter. Once separated by electrophoresis the desired fragments containing the fusion joints were excised from the gel and purified. Two methods of purification were tried and the Gene Clean kit (section 2.39.1(b)) was found to result in a smaller loss of fragment than electroelution (section 2.39.1(a)).

The purified *Pst*I/*Eco*RI fragments containing the fusion joints from each plasmid were ligated into *Pst*I/*Eco*RI restricted M13mp19 vector DNA as described in section 2.39.2.1. After overnight ligation, competent *E. coli* TG1 cells (section 2.37.2), were transformed with between 1-3 μl of ligation mix and the subsequent lawns on agar plates containing 5-bromo-4-chloro-3-indoxyl β-D-galactoside (X-gal), examined for plaque formation (section 2.39.2.2). M13 vectors produce the α-peptide of β-galactosidase which complements the *lac* deletion mutation in *E. coli* strains such as TG1 (table 2.1). When such strains are transfected by non-recombinant vectors, blue plaques are generated on plates containing X-gal. Vectors with inserts give rise to colourless plaques.

Small clear plaques were isolated and the viral DNA from these was propagated in *E. coli* TG1 and purifed (section 2.39.2.3). The large fragment of *E. coli* DNA polymerase I (Klenow enzyme) was used to sequence across the fusion joint of single stranded DNA, primed with a 19 bp portion of the IS50L of Tn5 ($^{5'}$CAGGACGCTACTTGTGTAT^{3}), as described in sections 2.39.2.4 & 2.39.4.

Figure 5.13 Agarose gel electrophoresis of *Pst*I and *Eco*RI restricted plasmids pSLF22 and pSLF34

Plasmids were restricted as indicated and electrophoresed on a 0.7% (w/v) agarose gel at 30V overnight in 1 x TBE and stained with ethidium bromide. Fusion joint containing fragments are arrowed : (1) = pSLF22, (2) = pSLF34.



Key to tracks
A : Phage lambda DNA restricted with *Hind*III.
B&C: pSLF22 & pSLF34 respectively - *Pst*I restricted.
D&E: pSLF22 & pSLF34 respectively - *Pst*I/*Eco*RI restricted.

*Pst*I restriction of pSLF22 produces fragment sizes : 0.92, 1.05, 2.36, 2.7 & 9.8 Kb (track B). *Pst*I/*Eco*RI restriction of pSLF22 results in digestion of the 2.7 & 9.8 Kb *Pst*I fragments to fragments of size : 4.24, 3.3, 2.27, 1.5 & 0.33 Kb. Additionally the *Pst*I/*Eco*RI fusion joint containing fragment comigrates with the 0.92 Kb *Pst*I fragment (track D).

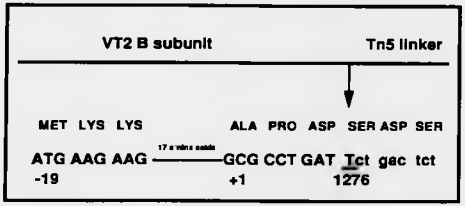
*Pst*I restriction of pSLF34 produces fragment sizes : 0.92, 1.05, 2.36, 2.9 & 9.4 Kb (track C). *Pst*I/*Eco*RI restriction of pSLF34 results in digestion of the 2.9 & 9.4 Kb *Pst*I fragments to fragments of size : 4.24, 3.3, 2.07, 1.5 & 0.33 Kb. Additionally the *Pst*I/*Eco*RI fusion joint containing fragment comigrates with the 1.05 Kb *Pst*I fragment (track E).

Despite numerous attempts, sequencing by this method proved unsuccessful, no clear sequence being obtained. This could either be due to lack of template DNA or a primer site deletion (Bankier *et al.*, 1986). Subsequent sequence analysis of recombinant vector DNA using a 17 base M13 primer ($^{5'}$GTTTTCCCAGTCACGAC$^{3'}$) provided by Don Cardy, Warwick University, showed the clones sequenced to consistently correspond to M13 deletions. M13 has been shown to be an unstable vector for inserts much greater than 2 Kb and some sequences, often highly repetitive, clone very rarely or are deleted readily from M13 (Bankier *et al.*, 1986). In this study the size of insert DNA was within the range expected to produce stable recombinants (0.92 & 1.03 Kb for pSLF22 & pSLF34 respectively), and the reason for the inability to obtain stable M13 recombinants was unclear.

Direct plasmid sequencing (section 3.39.3) proved to be more successful. The Tn*5* primer was end-labelled (section 2.39.3.1) and denatured plasmid DNA (section 2.39.3.2) sequenced using the Sequenase Version 2.0 kit (US Biochemical Corp.) as described in section 2.39.3.3. Sequenase Version 2.0 is derived from bacteriophage T7 DNA polymerase and has been genetically altered to have no 3'-5' exonuclease activity. It's properties include high processivity with less background radioactivity and a more uniform band intensity. Manganese buffer, provided in the sequenase kit, was added to the reaction mix prior to addition of the sequenase enzyme as this leads to a reduction in the average length of DNA synthesised in the termination step, intensifying bands corresponding to the sequences close to the primer. Sequence analysis of the resulting autoradiographs enabled the site of insertion of Tn*phoA* within the *slt*-II operon of pSLF22 to be obtained. Comparison with the published sequence data for VT2 (Jackson *et al.*, 1987b) showed that Tn*phoA* had inserted in-frame, in the B subunit gene adjacent to nucleotide 1276. This position was 10 bp downstream of the signal peptide cleavage site (figure 5.14), and 129 bp downstream of the *Pst*I cleavage site. Attempts to sequence the fusion joint in pSLF34 were unsuccessful and could have been due to

impurities in the plasmid preparation. Plasmid pSLF22 was subsequently selected for use in the study of VT2 gene expression.

Figure 5.14 **Sequence analysis of the insertion site of Tn*phoA* into *slt*II in plasmid pSLF22.**



The sequence shows the beginning of the B subunit gene of *slt*-II and the insertion site of Tn*phoA* (arrowed) in *slt*-IIB. The last base pair of *slt*-IIB is underlined with the deduced *slt*-II amino-acid sequence shown above the line and the N-terminal alanine of the processed B polypeptide depicted as +1.

5.2.4 Cytotoxicity assays of *E. coli* CC118 (*slt*-II::Tn*phoA*) gene fusion strains

Polymyxin B extracts of *E. coli* strains E3787, E32511, 60R363, CC118 (pSLF22) and CC118 (pSLF34) were prepared (section 2.21.1) and assayed for cytotoxicity using the Ricin assay as previously described (section 2.21.2). All strains produced active A subunit, evidenced by a distinctive aniline releasable fragment (figure 5.15). When Vero cell monolayers (section 2.21.3) were challenged with polymyxin B cell extracts of *E. coli* strains O157:H7 (ATCC 35150), DH1, 60R363, CC118(pSLF22) and CC118 (pSLF34) however, only the monolayers which had been challenged with polymyxin B cell extracts from *E. coli* 60R363 and O157:H7 (ATCC 35150) were observed to be cytotoxic, causing destruction and death of the cells in the monolayer. In comparison, monolayers which had been challenged with polymyxin B cell extracts from *E. coli* DH1, CC118 (pSLF22) and CC118 (pSLF34) remained intact and there was no significant evidence of cytotoxicity, even after 48 hours incubation. This evidence supports the results obtained in section 5.2.2 that insertion of Tn*phoA* had occurred in the B subunit of *slt*-II in plasmids pSLF22 and pSLF34. Active PhoA is produced indicating that translation of the *slt*-II B subunit occurs, however, insertion of Tn*phoA* into the B subunit not unexpectedly disrupts B subunit function (i.e. attachment of the holotoxin to the target cell). The A subunit can still exhibit N-glycosidase activity in a cell-free system such as the Ricin assay, where A subunit delivery by the B subunit to the target site is not required.

5.3 Homologous recombination of *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* gene fusions into the chromosome

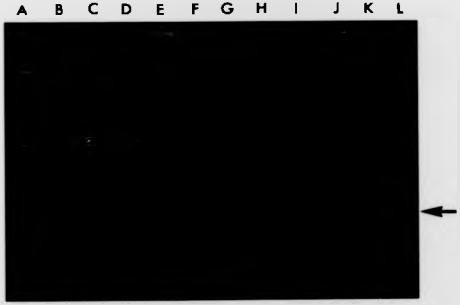
The fusion of Tn*phoA* to the *slt*-IA subunit gene (Calderwood & Mekalanos, 1987), and to the *slt*-IIB subunit gene (this study) allows measurement of expression of the *slt*-I and *slt*-II operons under different growth conditions by assay of PhoA activity. Although many studies have involved the use of multicopy fusion plasmids, plasmid copy number

Figure 5.15 Analysis of yeast ribosomal RNA treated with *E. coli* CC118 (pSLF22) and CC118 (pSLF34) polymyxin B cell extracts

Electrophoresis of RNA was performed on a 1.2% (w/v) agarose, 50% (v/v) formamide gel. The gel was electrophoresed at 20 mA constant current in Leicester Biocenter gel tanks for 2-3 hours in 0.1 x TPE buffer, stained in 2 μg ml^{-1} ethidium bromide for 30 minutes and destained in distilled water for 30 minutes.

Key to tracks

A : Yeast rRNA treated with recombinant ricin A chain (- aniline).
B : Yeast rRNA treated with recombinant ricin A chain (+ aniline).
C : Yeast rRNA treated with E3787 polymyxin B cell extract (- aniline).
D : Yeast rRNA treated with E3787 polymyxin B cell extract (+ aniline).
E : Yeast rRNA treated with E32511 polymyxin B cell extract (- aniline).
F : Yeast rRNA treated with E32511 polymyxin B cell extract (+ aniline).
G : Yeast rRNA treated with 60R363 polymyxin B cell extract (- aniline).
H : Yeast rRNA treated with 60R363 polymyxin B cell extract (+ aniline).
I : Yeast rRNA treated with CC118 (pSLF22) polymyxin B cell extract (- aniline).
J : Yeast rRNA treated with CC118 (pSLF22) polymyxin B cell extract (+ aniline).
K : Yeast rRNA treated with CC118 (pSLF34) polymyxin B cell extract (- aniline).
L : Yeast rRNA treated with CC118 (pSLF34) polymyxin B cell extract (+ aniline).



has been shown to vary considerably with such parameters as promoter strength and cellular growth conditions (Adams & Hatfield, 1984). It is therefore desirable to have single copy derivatives of the *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* fusions to eliminate the problems associated with uncontrolled fluctuations in plasmid copy number which affect gene dosage. In the 1987 study of iron regulation of the *slt*-I operon using pSC105, Calderwood and Mekalanos accounted for this by determining β-lactamase activity, encoded for by the Ap^r gene on plasmid pSC105, in parallel with PhoA activity. Plasmid pSLF22 does not have such an assayable activity and as there has been no report of plasmid encoded VT production, attempts were made to more closely mimic the *in vivo* condition by introducing fusion plasmids pSC105 and pSLF22 into *E. coli* strains E3787 and E32511 respectively. To achieve single copies, homologous recombination between the gene fusions on the plasmid and the equivalent regions on the chromosome of the wild type *E. coli* strains was then attempted.

5.3.1 Transformation of *E. coli* E3787 and E32511 with plasmids pSC105 and pSLF22 respectively

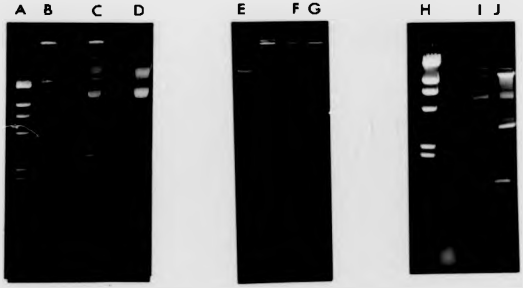
E. coli strains E3787 and E32511 were selected to act as the recipients for homologous recombination of *slt*::Tn*phoA* gene fusions. Sensitivity of E3787 to Ap and Kn and E32511 to Tc and Kn was confirmed by plating the strains onto the appropriate plates. Caesium chloride gradient pure pSC105 and pSLF22 (section 2.13.1(b)) were electroporated into the host strains E3787 and E32511 respectively (section 2.37.1) and transformants selected according to the drug resistance markers of the recombinant plasmids. Presence of plasmid from a single transformant of each was confirmed by small scale plasmid preparation as described in section 2.13.2, (figure 5.16). In track C it can be seen that the additional plasmid bands in the E3787 (pSC105) transformant comigrate with caesium chloride gradient purified pSC105 (track D), whilst *Eco*RV restriction of plasmid DNA from the E32511 (pSLF22) transformant showed the four distinctive *Eco*RV bands indicative of presence of pSLF22 (tracks I & J respectively).

Figure 5.16 **Agarose gel electrophoresis of plasmid DNA from *E. coli* E3787 and E32511 before and after electroporation with pSC105 and pSLF22 respectively**

Plasmid DNA was restricted as indicated and electrophoresed on a 0.7% (w/v) agarose gel at 40mA in 1 x TBE and stained with ethidium bromide.

Key to tracks

A,E & H :	Phage lambda DNA restricted with *Hind*III.
B :	Plasmid DNA from E3787 - unrestricted.
C :	Plasmid DNA from E3787 (pSC105) - unrestricted.
D :	CsCl density gradient purified pSC105 - unrestricted.
F :	Plasmid DNA from E32511 - unrestricted.
G :	Plasmid DNA from E32511 (pSLF22) - unrestricted.
I :	Plasmid DNA from E32511 - *Eco*RV restricted.
J :	Plasmid DNA from E32511 (pSLF22) - *Eco*RV restricted.



Permeabilised cells (section 2.44.1(b)) were assayed for PhoA activity as described in section 2.44.2, before and after electroporation with pSC105 and pSLF22 (table 5.1). Background levels of endogenous activity for *E. coli* strains E3787 and E32511 were very low and found to increase 143 and 86 fold in E3787 and E32511 respectively, indicating that pSC105 and pSLF22 had been successfully introduced.

5.3.2 Low phosphate treatment of E3787 (pSC105) and E32511 (pSLF22)

The approach taken to create single copy derivatives was based on destabilising pSC105 and pSLF22 such that they were lost from the bacterial cell. By selecting for Kn^r during the process, homologous recombination should occur between the sequences on the plasmid and the equivalent regions on the *E. coli* chromosome to leave a single copy of the gene fusion within the cell.

An assessment of the relative plasmid stabilities within E3787 and E32511 (section 2.40) showed that both plasmids were stably maintained under normal growth conditions, with no significant loss from the population when some degree of selective pressure was applied. With no selective pressure imposed pSC105 was still found in 100% of the viable cell population, whilst 85% of the population still carried pSLF22. This stable maintenance is not unexpected as under normal growth conditions the high plasmid copy number of pSC105 and pSLF22 ensures continued distribution among the daughter cells upon segregation. Under limiting growth conditions however, plasmid copy number is reduced, thus diminishing the probability that a plasmid will be distributed to both daughter cells during cell division (Roeder & Collmer, 1985). Roeder & Collmer (1985) have shown that plasmid pBR322 is rendered unstable under phosphate limitation and utilised this phenomenon to obtain high frequencies of exchange recombination in *Erwinia chrysanthemi* carrying a *pelC*::Kn mutation on pBR322. A similar approach was adopted in this study to construct single copy derivatives of pSC105 and pSLF22.

Plasmid curing was performed by growing the transformants on low phosphate media for 48 and 96 hours at 37^{o}C and selecting for maintenance of Kn^{r} (section 2.41). After each 2 day incubation in phosphate limited media, serial dilutions were plated onto LB + Kn and LB + Ap or LB + Tc plates as appropriate. The results are summarised in table 5.2 where it can be seen that after one round of plasmid curing approximately 70% of E3787 and 85% of E32511 transformants were Kn^{r} but Ap or Tc sensitive (Ap^{S} or Tc^{S}) respectively. After a second round of plasmid curing this rose to 99.6% for E3787 and 97.5% for E32511.

After the second round of plasmid curing, 50 colonies displaying Ap or Tc sensitivity were patched onto LB + Kn plates to confirm plasmid loss, rather than that a deletion or mutation of the plasmid-borne antibiotic resistance genes had occurred. All were found to display Kn^{r}. Similarily 50 colonies displaying Kn^{r} were patched onto Ap or Tc plates as appropriate and all were found to display Ap^{S} or Tc^{S}.

Colonies displaying Kn^{r} could either have occurred as a result of exchange recombination or by transposition of Tn*phoA* into the chromosome of the *E. coli* strain. Obvious differences were apparent in the relative PhoA activity of the Kn^{r}/Ap^{S} and Kn^{r}/Tc^{S} colonies on plates containing XP, with some colonies appearing much bluer (i.e. more PhoA active) than others. There are two possible explanations for this:-

(1) Colonies displaying higher PhoA activity represent homologous recombinants or strains in which transposition of Tn*phoA* to an exported target gene in the chromosome has occurred.

(2) Colonies displaying limited PhoA activity represent homologous recombinants or strains in which transposition of Tn*phoA* to a non-exportable target gene has occurred.

It is therefore necessary to confirm whether, and in which colonies, exchange recombination has in fact occurred. This was performed by Western blotting gene fusion products with PhoA antibody (section 2.43) and Southern blotting potential

Table 5.1 Alkaline phosphatase activity of *E. coli* strains grown on Luria Bertani media

The *E. coli* strains indicated were grown overnight on LB at 37°C with shaking (250 rpm). The OD600nm of each culture was taken and cells permeabilised and assayed for PhoA as described in sections 2.44.1(b) & 2.44.2. Units of PhoA activity are normalised to the OD600nm of the bacterial culture

Strain	PhoA activity (U)
E. coli E3787	1.5
E. coli E32511	1.4
E. coli CC118 (pSC105)	270.1
E. coli CC118 (pSLF22)	87.1
E. coli E3787 (pSC105)	214.8
E. coli E32511 (pSLF22)	120.9

Table 5.2 Low phosphate treatment of E. coli E3787 (pSC105) and E32511 (pSLF22)

Following 48 hours (round 1), and 96 hours (round 2), growth in low phosphate medium containing kanamycin, cultures were serially diluted in phosphate buffered saline and spread plated onto selective agar plates as indicated. Ap^r or Tc^r colonies were taken to represent E3787 and E32511 cells that had retained plasmids pSC105 and pSLF22 respectively

	Viable Count ($cfu\ ml^{-1}$)		
Strain	LB + Km	LB + Ap	LB + Tc
Round 1			
E3787 (pSC105)	6.5×10^8	1.91×10^8	-
E32511 (pSLF22)	4.7×10^8	-	7.3×10^7
Round 2			
E3787 (pSC105)	3.8×10^8	1.6×10^6	-
E32511 (pSLF22)	6.7×10^8	-	1.7×10^7

homologous recombinants to determine whether insertion of Tn*phoA* into the chromosomal *slt* operon had occurred.

5.3.3 Western blotting of gene fusion products

The point of insertion of Tn*phoA* is 85 nucleotides (28 amino acids) and 10 nucleotides (3 amino acids) downstream from the signal sequence cleavage site in pSC105 (Calderwood & Mekalanos, 1987) and pSLF22 (section 5.2.3) respectively. Assuming that the average molecular weight (MW) of one amino acid residue is 110 daltons (Boquet *et al.*, 1987), the *phoA* gene in Tn*phoA* encodes a protein of 450 amino acids, and correct processing of the signal peptide takes place, the size of the *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* fusion proteins can be predicted as being 52.58 KDa & 49.83 KDa respectively.

As the size of the chimeric protein produced by the *slt*-II::Tn*phoA* gene fusion is not significantly larger than native alkaline phophatase (MW 47 KDa) it was anticipated that there might be a problem in differentiating this protein from non-*slt*-II::Tn*phoA* gene fusion products of similar size that might occur upon transpositon of Tn*phoA* into the chromosome. In addition, degradation of gene fusion products to native PhoA has been previously reported by Hoffman & Wright (1985). To overcome this problem and as a more rapid screen for potential homologous recombinants, mitomycin C (final concentration 1 μg ml^{-1}), which has been shown to increase VT1 and VT2 production (section 1.4.4.5), was added to exponentially growing cultures of the single copy candidates in LB. After overnight incubation, periplasmic proteins were released from the bacterial cells in response to cold osmotic shock treatment as described in section 2.42. Samples adjusted to contain the same amount of protein were then separated by SDS PAGE, transferred to nitrocellulose and probed with antibody to PhoA as described in section 2.43.1. Antigen-antibody complexes were subsequently detected using a horse radish peroxidase colour reaction (section 2.43.2). *Slt*::Tn*phoA*

homologous recombinants were inferred when increased expression of a PhoA containing protein in the presence of mitomycin C was observed in comparison to a control of the same culture grown on LB alone.

Figure 5.17 shows the Western blots obtained from single copy fusion candidates after growth with or without mitomycin C (gel A & gel B respectively). Purified bacterial PhoA (*E. coli* type IIIL, Sigma) was added as a control and it can be seen that CC118, a *phoA* mutant, does not produce PhoA as expected. *E. coli* strains E3787 and E32511 also appear to be only very slightly PhoA positive. Low MW proteins that cross-react with the antibacterial PhoA antibody are evident in all tracks except 7 & 19. These are taken to represent proteolysis or degradation products that have arisen during sample preparation. It is immediately apparent that expression of *phoA* is under the control of a mitomycin C inducible promoter in *E. coli* E32511 *slt*-II::Tn*phoA* single copy fusion candidates 1 & 2. None of the other single copy fusion candidates in figure 5.17 appear to be mitomycin C inducible. *E. coli* CC118 harbouring plasmids pSLF22 and pSC105 is not mitomycin C inducible as the plasmids contain only part of the cloned *slt* operons and not the phage sequences responsible for mitomycin C induction. High MW proteins are evident, especially in tracks 1 & 2, that comigrate with the 94 KDa protein marker. These might possibly represent PhoA containing holotoxin that has not completely dissociated under the denaturing conditions employed. Further Western analysis of potential E3787 *slt*-I::Tn*phoA* single copy fusion candidates failed to show evidence of mitomycin C induction (results not shown).

Iron limitation has been shown to increase expression of VT1 but not VT2 (section 1.4.4.3). If VT1 expression was not under the control of phage sequences in potential single copy derivatives, this would explain why no mitomycin C induction was observed. To rule out this possibility, it was decided therefore, to screen for potential *slt*-I::Tn*phoA* homologous recombinants under iron limiting conditions. Cultures were grown overnight in iron limited LB (section 2.3.2) and Western analysis of periplasmic

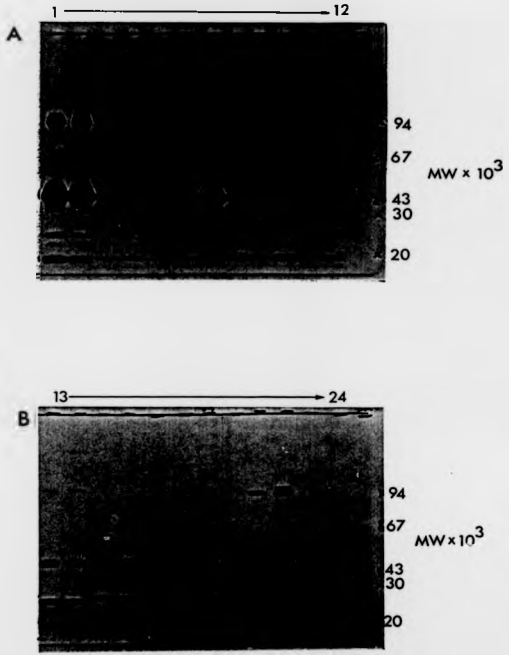

A
1
12
94
67
43
30
20
MW x 10^3
B
13
24
94
67
43
30
20
MW x 10^3

Figure 5.17 Western analysis of *E. coli* E3787 (*slt*I::Tn*phoA*) and E32511 (*slt*II::Tn*phoA*) single copy fusion candidates

Strains were grown on LB with or without mitomycin C (final concentration 1 $\mu g\ ml^{-1}$) as indicated. Periplasmic protein preparations (section 2.42), adjusted to contain 30 μg of protein were separated by electrophoresis through an 11% (w/v) linear SDS polyacrylamide gel and immunoblotted with rabbit antialkaline phophatase antibody (sections 2.43.1 & 2.43.2).

Key to tracks

Blot A (mitomycin C added)

1 *E. coli* E32511 *slt*II::Tn*phoA* single copy fusion candidate 2
2 *E. coli* E32511 *slt*II::Tn*phoA* single copy fusion candidate 1
3 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 4
4 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 3
5 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 2
6 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 1
7 *E. coli* alkaline phosphatase
8 *E. coli* CC118
9 *E. coli* CC118 harbouring pSLF22
10 *E. coli* CC118 harbouring pSC105
11 *E. coli* E32511
12 *E. coli* E3787

Blot B

Tracks 13-24 correspond to tracks 1-12 respectively except that samples were from strains grown in LB without mitomycin C.

proteins performed as described above. Expression of PhoA fusions were compared to cultures grown on LB (i.e. iron replete conditions). In figure 5.18 it can be seen that expression of plasmid encoded *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* gene fusions increased under iron limitation. Calderwood & Mekalanos (1987) reported a 13-16 fold increase in PhoA activity for *E. coli* CC118 (pSC105) grown under iron limited conditions. Iron regulation of VT2 has not been reported however, and it is likely that plasmid copy number fluctuations as a result of the physiological constraint applied could have led to an increase in the respective gene dosages in this case. Lack of VT2 induction under iron limitation in potential *slt*-II::Tn*phoA* homologous recombinants 1 & 2 supports this hypothesis. Of all the potential VT1 single copy derivatives tested, only three (24, 25, & 45) appeared to be induced under iron limitation (figure 5.19 & 5.20).

As a consequence of this work, VT1 single copy fusion candidates 24, 25 & 45 and VT2 single copy fusion candidates 1 & 2 were selected for Southern analysis to confirm whether insertion of Tn*phoA* into the chromosomally encoded *slt*-I and *slt*-II operons had occurred.

5.3.4 Southern blot analysis of single copy fusion candidates

Figure 5.21 shows the results obtained when 10 μg of chromosomal DNA (section 2.12(a)), prepared from *E. coli* E32511 and the *slt*-II::Tn*phoA* single copy fusion candidates 1 & 2 were digested with *Eco*RV, electrophoresed on a 0.6% (w/v) agarose gel, Southern blotted and probed with freshly prepared ^{32}PdCTP labelled VT2 probe (sections 2.18 & 2.19). E32511 contains two *slt* operons, *slt*-II and *slt*-IIc (section 3.5.2) and two *Eco*RV fragments of estimated size 6.4 and 5.6 Kb hybridised with the VT2 probe (tracks 10 & 13). In both single copy fusion candidates 1 & 2, the 6.4 Kb fragment increased in size to approximately 14.5 Kb, indicating that insertion of Tn*phoA* had occurred (tracks 11, 12 & 14, 15). As it was unknown whether the 6.4 Kb *Eco*RV fragment corresponded to *slt*-II or *slt*-IIc coding sequence, 10 μg of each chromosomal

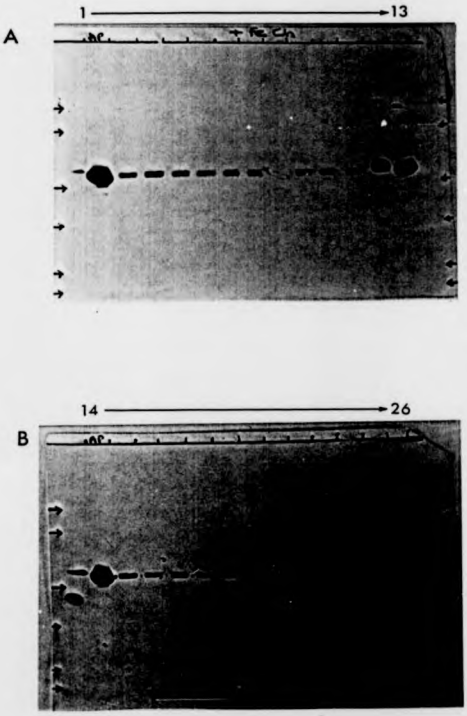

A
1
13
B
14
26

Figure 5.18 Western analysis of single copy fusion candidates grown under iron limited and iron replete conditions

Strains were grown on LB with or without 0.2 mM 2,2' dipyridyl (Sigma) as indicated. Periplasmic protein preparations (section 2.42), adjusted to contain 15 μg of protein were separated by electrophoresis through an 11% (w/v) linear SDS polyacrylamide gel and immunoblotted with rabbit antialkaline phophatase antibody (sections 2.43.1 & 2.43.2).

Key to tracks

Blot A (iron limited LB)

1 *E. coli* alkaline phosphatase
2 *E. coli* E32511 *slt*II::T*phoA* single copy fusion candidate 2
3 *E. coli* E32511 *slt*II::T*phoA* single copy fusion candidate 1
4 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 28
5 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 18
6 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 16
7 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 14
8 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 11
9 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 5
10 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 3
11 *E. coli* E3787 *slt*I::T*phoA* single copy fusion candidate 2
12 *E. coli* CC118 harbouring pSLF22
13 *E. coli* CC118 harbouring pSC105

Blot B

Tracks 14-26 correspond to gel A tracks 1-13 respectively grown in iron replete LB.

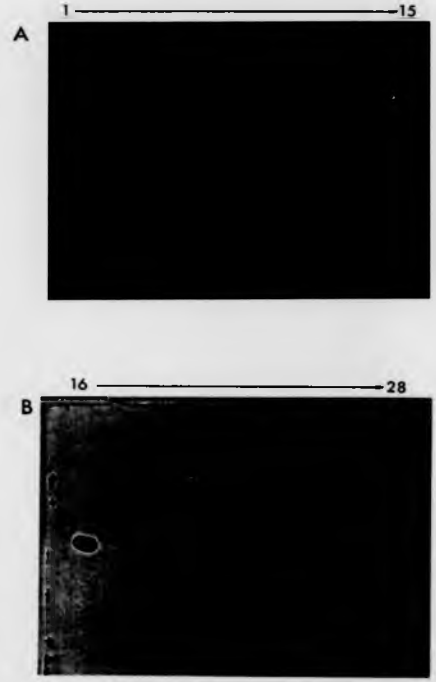

A
1
15
B
16
28

Figure 5.19 Ponceau S and chloronaphthol stained Western blot of single copy fusion candidates grown under iron replete conditions

The strains indicated were grown on LB and periplasmic protein preparations (section 2.42), adjusted to contain 15 μg of protein, separated by electrophoresis through an 11% (w/v) linear SDS polyacrylamide gel and immunoblotted with rabbit antialkaline phophatase antibody (sections 2.43.1 & 2.43.2).

Key to tracks

Blot A (Ponceau S stained)

1&15 Protein markers
2 *E. coli* alkaline phosphatase
3 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 45
4 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 42
5 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 25
6 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 24
7 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 13
8 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 12
9 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 10
10 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 9
11 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 8
12 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 7
13 *E. coli* E3787 *slt*I::Tn*phoA* single copy fusion candidate 6
14 *E. coli* CC118 harbouring pSC105

Blot B

Tracks 16-28 correspond to gel A tracks 2-14 respectively after probing with PhoA antibody and detection of antigen-antibody complexes using a horseradish peroxidase colour reaction.

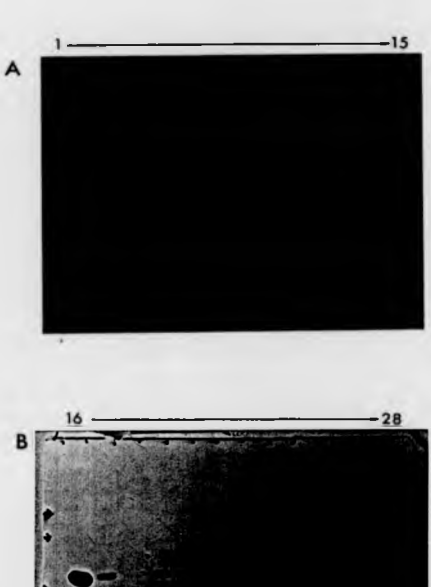

A
1
15
B
16
28

Figure 5.20 Ponceau S and chloronaphthol stained Western blot of single copy fusion candidates grown under iron limited conditions

The strains indicated were grown on LB containing 0.2 mM 2, 2' dipyridyl (Sigma) and periplasmic protein preparations (section 2.42), adjusted to contain 15 μg of protein, separated by electrophoresis through an 11% (w/v) linear SDS polyacrylamide gel and immunoblotted with rabbit antialkaline phophatase antibody (sections 2.43.1 & 2.43.2).

Key to tracks

Blot A (Ponceau S stained)

1&15 Protein markers

2 *E. coli* alkaline phosphatase

3 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 45

4 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 42

5 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 25

6 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 24

7 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 13

8 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 12

9 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 10

10 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 9

11 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 8

12 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 7

13 *E. coli* E3787 *sltI*::Tn*phoA* single copy fusion candidate 6

14 *E. coli* CC118 harbouring pSC105

Blot B

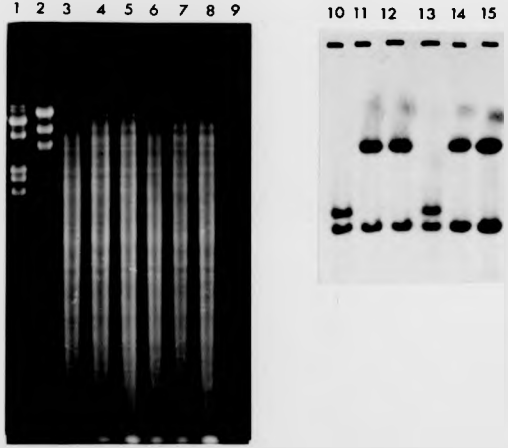
Tracks 16-28 correspond to gel A tracks 2-14 respectively after probing with PhoA antibody and detection of antigen-antibody complexes using a horseradish peroxidase colour reaction.

Figure 5.21 **Southern analysis of *Eco*RV digested *E. coli* E32511 *slt*-II::Tn*phoA* single copy fusion candidates 1 & 2**

Chromosomal DNA was digested with *Eco*RV and the products size-fractionated by 0.6% (w/v) TBE agarose gel electrophoresis at 100V. DNA was transferred to nitrocellulose and probed with a ^{32}PdCTP labelled 0.85 Kb *Sma*I-*Pst*I fragment from plasmid NTP707 that encodes the *slt*-IIA gene sequence.

Key to tracks

1 : Lambda DNA digested with *Bam*HI.
2 : Lambda DNA digested with *Bgl*II.
3&6: *E. coli* E32511 chromosomal DNA digested with *Eco*RV.
4&7: Single copy fusion candidate 1 chromosomal DNA digested with *Eco*RV.
5&8: Single copy fusion candidate 2 chromosomal DNA digested with *Eco*RV.
9 : Lambda DNA restricted with *Hind*III.



Tracks 10-15 is the autoradiograph of corresponding tracks 3-8 after washing in 1 x SSC, 0.1% (w/v) SDS at 65^{o}C and 2 days exposure at -70^{o}C.

NB DNA size markers produced by lambda DNA digestion
*Bam*HI - 16.8, 7.2, 6.8*, 6.5, 5.6* & 5.5 Kb.
*Bgl*II - 22, 13.3, 9.7, 2.4, 0.65, 0.42 & 0.06 Kb.
*Hind*III - 23.6, 9.46, 6.78, 4.34, 2.26, 1.98 & 0.56 Kb.
*Fragments have cos ends and can reassociate to form additional bands.

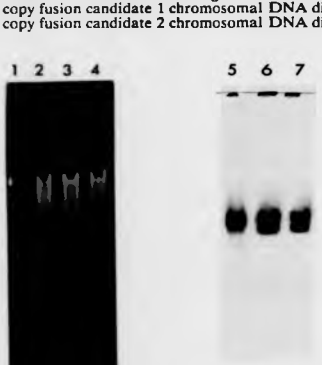
DNA preparation was digested to completion with *Eco*RI, electrophoresed on a 1% (w/v) agarose gel and Southern blotted as before. The resulting autoradiograph is shown in figure 5.22. In figure 5.21 two *Eco*RI fragments, of estimated size 5.8 & 5.2 Kb, hybridise with the VT2 probe. These correspond to the 4.9 Kb *slt*-IIc and 4.3 Kb *slt*-II operons respectively (table 3.3). In the single copy fusion candidates 1 & 2 however, the 5.2 Kb *Eco*RI fragment has decreased in size to approximately 5.0 Kb indicating that exchange recombination had occurred into the *slt*-II operon and not the *slt*-IIc operon in E32511. The *E. coli slt*-II::Tn*phoA* single copy fusion candidates 1 & 2 were designated E. coli E32511 (SLF22/1) and E32511 (SLF22/2) respectively.

Chromosomal DNA was also prepared from *E. coli* E3787 and the *slt*-I::Tn*phoA* fusion candidates 24, 25 & 45. The non-iron inducible single copy fusion candidate 42 was also included for comparison purposes. 10 μg of DNA sample was digested to completion with *Eco*RV, electrophoresed on a 0.7% (w/v) agarose gel and the resulting Southern blot probed with freshly prepared ^{32}PdCTP labelled VT1 probe (figure 5.23). In all the strains a 9.3 Kb fragment hybridised with the VT1 probe. This indicated that Tn*phoA* insertion into the chromosomally encoded *slt*-I operon, had not occurred in any of the single copy fusion candidates tested. Single fusion candidates 24, 25, 42 & 45 appeared much more PhoA active on plates containing XP. From this it was concluded that single copy fusion candidate 42 represented a strain in which transposition of Tn*phoA* had occurred to a chromosomally encoded exported protein that was not iron inducible, whereas single copy fusion candidates 24, 25, 45 represent transpositions of Tn*phoA* to iron inducible exported proteins.

Figure 5.22 **Southern analysis of *Eco*RI digested *E. coli* E32511 *slt*-II::Tn*phoA* single copy fusion candidates 1 & 2**

Chromosomal DNA was digested with *Eco*RI and the products size-fractionated by 1% (w/v) TBE agarose gel electrophoresis at 100V. DNA was transferred to nitrocellulose and probed with a ^{32}PdCTP labelled 0.85 Kb *Sma*I-*Pst*I fragment from plasmid NTP707 that encodes *slt*-IIA gene sequence.

Key to tracks
1 : Lambda DNA digested with *Hind*III.
2 : *E. coli* E32511 chromosomal DNA digested with *Eco*RI.
3 : Single copy fusion candidate 1 chromosomal DNA digested with *Eco*RI.
4 : Single copy fusion candidate 2 chromosomal DNA digested with *Eco*RI.



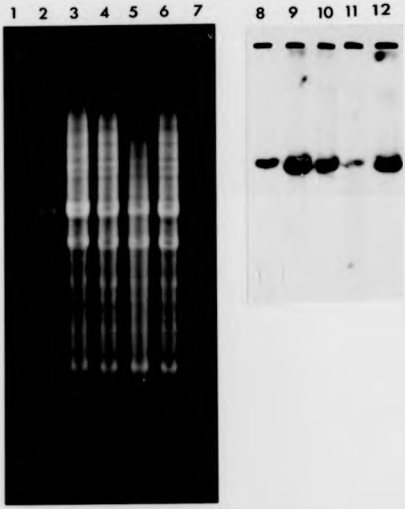
Tracks 5-7 is the autoradiograph of corresponding tracks 2-4 after washing in 0.1 x SSC, 0.1% (w/v) SDS at 65^{0}C and 30 hours exposure at -70^{0}C.

Figure 5.23 Southern analysis of *Eco*RV digested *E. coli* E3787 *slt*-I::Tn*phoA* single copy fusion candidates 24, 25, 42 & 45

Chromosomal DNA was digested with *Eco*RV and the products size-fractionated by 0.7% (w/v) TBE agarose gel electrophoresis at 20V overnight. DNA was transferred to nitrocellulose and probed with a ^{32}PdCTP labelled 0.75 Kb *Hinc*II fragment from plasmid NTP705 that encodes the *slt*-IB gene sequence.

Key to tracks

1&7: Lambda DNA digested with *Hind*III.
2 : *E. coli* E3787 chromosomal DNA digested with *Eco*RV.
3 : Single copy fusion candidate 24 chromosomal DNA digested with *Eco*RV.
4 : Single copy fusion candidate 25 chromosomal DNA digested with *Eco*RV.
5 : Single copy fusion candidate 42 chromosomal DNA digested with *Eco*RV.
6 : Single copy fusion candidate 45 chromosomal DNA digested with *Eco*RV.



Tracks 8-12 is the autoradiograph of corresponding tracks 2-6 after washing in 0.1 x SSC, 0.1% (w/v) SDS at 65^{0}C and 4 days exposure at -70^{0}C.

5.4 Summary and overview

The initial sections of this chapter have described the successful fusion of the *phoA* gene to the *E. coli slt*-IIB structural gene so that expression of the former is brought under the control of the *slt*-II regulatory regions. Following from this work, the *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* gene fusion vectors pSC105 and pSLF22 were introduced into wild type *E. coli* strains E3787 and E32511 respectively by electroporation The ultimate aim was to construct single copy fusion derivatives of pSC105 and pSLF22 via exchange-recombination to enable studies on the regulation of the *slt*-I and *slt*-II operons.

Evidence (i.e. loss of plasmid marker, retention of Kn resistance, mitomycin C induction and disruption of the *slt*-II operon) strongly suggest that single copy fusion derivatives of pSLF22 had been constructed. These were termed SLF22/1 and SLF22/2. A single copy fusion derivative of pSC105 however, was not obtained in this study under low phosphate conditions. Although loss of plasmid marker and retention of Kn resistance was demonstrated after growth under phosphate limitation, mitomycin C induction and disruption of the *slt*-I operon was not apparent in any of the single copy fusion candidates studied. Plasmid copy number fluctuations of pSC105 however, can be monitored by assaying β-lactamase activity in association with PhoA (Calderwood & Mekalanos, 1987). *E. coli* strains CC118 (pSC105) and E32511 (SLF22/1) were therefore selected to form the basis of the following chapter which examines the effect of several physical parameters on *slt*-I and *slt*-II production.

CHAPTER SIX

6. Studies on expression of the *slt*-I and *slt*-II operons

6.1 Introduction

Major differences in the regulation of the *slt*-I and *slt*-II operons became apparent when transcription of the *slt*-I but not the *slt*-II operon was shown to dramatically increase under conditions of iron starvation (De Grandis *et al.*, 1987; section 1.4.4.3). As stated in section 1.10, this project was initiated to examine the physiology of VT production by *E. coli*. As a simple and sensitive means of monitoring VT2 expression, the transposon vector Tn*phoA* was subsequently used to create a fusion between the B subunit of the cloned *slt*-II operon and the gene for bacterial alkaline phophatase (chapter 5). Measurement of the PhoA activity of a single copy derivative of this fusion in strain E32511 (designated SLF22/1), and a plasmid encoded *slt*-I::Tn*phoA* gene fusion pSC105 (provided by S. B. Calderwood, Harvard University), enables rapid and quantitative assay of VT2 and VT1 expression respectively. This chapter describes the findings of experiments in which the *slt*::Tn*phoA* gene fusions were used to ascertain whether defined parameters affected VT1 and VT2 expression. PhoA activity was determined by measuring the rate of hydrolysis of p-nitrophenyl phosphate (Sigma 104), by permeabilised cells (section 2.44.1) and the data presented as a function of culture optical density at 600nm (OD600). To control for possible differences in plasmid copy number or a generalised increase in transcription of plasmid-encoded genes under the growth conditions examined, β-lactamase activity was determined in parallel with PhoA activity in strain CC118 (pSC105) by measuring the hydrolysis of nitrocephin (section 2.44.2). PhoA activities were also adjusted to account for background levels of endogenous activity for bacterial strains CC118 and E32511 where stated.

6.2 Expression of VT1 and VT2 during growth on Luria Bertani medium

To examine the expression of the *slt*-I and *slt*-II operons with respect to the growth phase of the bacterium, growth on LB was examined as described in section 2.45. The results are shown in table 6.1 and displayed graphically in figures 6.1 and 6.2.

Maximum toxin yields were present at 24 and 8 hours for VT1 and VT2 respectively and strain CC118 (pSC105) consistently produced higher levels of PhoA than E32511 SLF22/1. It has been observed that the cytotoxic activities in cell lysates of VT1-producing EHEC strains are 100 to 1000 fold higher than for VT2 or VTe producing strains (O'Brien & Holmes, 1987). In the present study however, at least part of the higher expression of VT1 is due to gene dosage associated with the multicopy plasmid in which the *slt*-I::Tn*phoA* gene fusion is cloned. During exponential growth of CC118 (pSC105) on LB (figure 6.1), VT1 synthesis occurred until the late exponential/early stationary phase (6-8 hours culture), when a sudden drop in the PhoA activity of the bacterial population was observed. This observation was consistently reproducible. The reason for the sudden drop in PhoA activity is unknown but may result from a decrease in the plasmid copy number as the β-lactamase activity also dropped suddenly at this point in growth (figure 6.4). There may also have been an increase in the turnover of the hybrid protein by cellular proteases at this time. As the culture entered stationary phase (8-10 hours culture) however, there appeared to be a sudden burst in VT1 synthesis, with continued synthesis for the next 14 hours. This could not be the result of an increase in the plasmid copy number as the β-lactamase activity was observed to steadily decrease during this time. After 48 hours growth VT1 synthesis appeared to have slowed as the total PhoA activity had hardly changed to that observed at 24 hours growth. In contrast, VT2 synthesis appeared to mimic growth (figure 6.2). There was an initial lag in VT2 production, followed by rapid synthesis throughout the exponential phase until the culture reached stationary phase, whereupon the cellular

Table 6.1 VT1 and VT2 expression during growth of *E. coli* CC118 (pSC105) and E32511 SLF22/1 on LB

(a) CC118 (pSC105)

Hours culture	PhoA activity (U) Cell	Sup	Total	ß-Lact activity (mU) Cell	Sup	Total
2	288.2	60.3	348.5	4105	772	4877
4	436.0	58.8	590.9	8798	1339	10137
8	330.7	63.6	394.3	7086	1631	8717
12	711.3	57.7	769.0	4852	1099	5951
24	1516.3	77.4	1593.7	43	1165	1208
48	1401.6	123.2	1524.8	50	1354	1404

(b) E32511 SLF22/1

Hours culture	PhoA activity (U) Cell	Sup	Total
2	17.5	1.7	19.2
4	24.7	0.38	25.1
6	81.0	0.99	82.0
8	84.0	12.9	96.9
12	57.1	32.4	89.5
24	49.7	29.9	79.6
48	46.3	33.4	79.7

The synthesis of *slt*I::Tn*phoA* and *slt*II::Tn*phoA* fusion proteins during growth on LB was determined as described in section 2.45. Cell extract and supernatant (Sup) samples were assayed for alkaline phosphatase (PhoA) and ß-Lactamase (ß-Lact), sections 2.44.2 & 2.44.3 respectively, in duplicate on two separate occasions. The figures represent the calculated mean activity from one experiment, corrected for background PhoA activity performed on *E. coli* CC118 (a) and E32511 (b) during growth.

Figure 6.1 Expression of VT1 during growth on Luria Bertani medium

VT1 expression during growth was determined by monitoring the synthesis of the VT1-PhoA hybrid protein produced by CC118 (pSC105), as described in the legend for table 6.1.

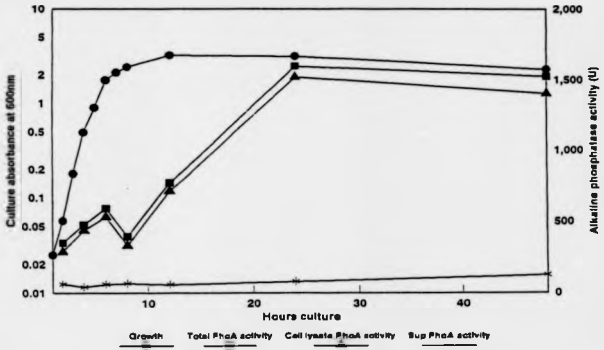


Figure 6.2 Expression of VT2 during growth on Luria Bertani medium

VT2 expression during growth was determined by monitoring the synthesis of the VT2-PhoA hybrid protein produced by E32511 SLF22/1, as described in the legend for table 6.1.

Figure 6.3 Extracellular VT1 and VT2 production during growth on Luria Bertani medium

VT1 and VT2 expression during growth was determined by monitoring the synthesis of the VT1-PhoA and VT2-PhoA hybrid proteins produced by CC118 (pSC105) and E32511 SLF22/1, as described in the legend for table 6.1. Extracellular VT1 and VT2 production during growth was determined by expressing the supernatant VT1-PhoA and VT2-PhoA activity as a percentage of the total VT1-PhoA and VT2-PhoA activity using the data shown in tables 6.1(a) and 6.1(b) respectively.

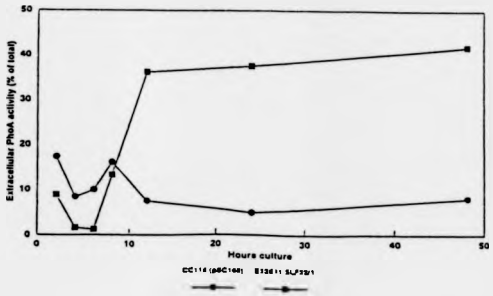
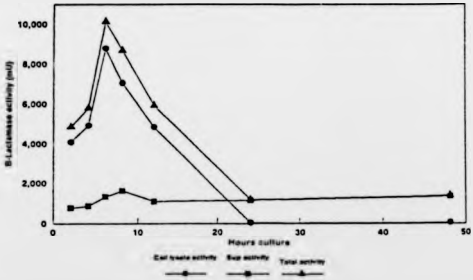


Figure 6.4 Expression of Beta-lactamase during growth of CC118 (pSC105) on Luria Bertani medium

ß-lactamase expression was determined during growth of CC118 (pSC105), as described in the legend for table 6.1 and plotted using data from table6.1(a).



VT2-PhoA activity suddenly decreased (8-10 hours culture). There appeared to be little turnover of the hybrid VT2-PhoA protein between 24 and 48 hours culture.

Translational fusions made by using Tn*phoA* can be used to gain information about the subcellular locations of the gene products by assaying the PhoA activity in cell fractions. Comparison of the culture supernatant PhoA activity as a percentage of the total PhoA activity of the two cultures indicated that there were significant differences in secretion and localisation of VT1 and VT2 in *E. coli* (figure 6.3). The VT1-PhoA hybrid protein was primarily cell associated throughout the growth cycle with the cell lysate containing between 83% to 95% of the total PhoA activity. This is in agreement with the findings of Weinstein *et al.* (1988b) who showed VT1 to be predominantly cell associated, with at least 75% of the total toxin cell associated. As the β-lactamase activity of the supernatant did not increase significantly in the stationary phase of growth (figure 6.4) it can be concluded that there was little cell lysis during the course of the experiment. A large proportion of the VT2-PhoA fusion protein on the otherhand was extracellularly located with at least 36% of the total PhoA activity present in the supernatant after 12 hours growth. This data correlates well with earlier work in the project which showed that VT1 predominates in cell lysates whilst VT2 is readily found in culture supernatants (section 3.6.3). Furthermore, the sudden decrease in cellular PhoA activity as the culture reached stationary phase coincided with the sudden production of the VT2-PhoA hybrid into the supernatant. This corroborates the observations made on the protein profiles of *E. coli* O157:H7 (ATCC 35150) in section 3.7. The appearance in the supernatant did not result from increased cell lysis and may have occurred as a result of leakage of VT2 across the outer membrane once the intracellular VT2 concentration reached a certain level.

6.3 Expression of VT1 and VT2 under iron limitation

Calderwood & Mekalanos (1987) developed plasmid pSC105 to enable study, at the molecular level, of the mechanism by which the iron concentration of the growth medium regulates the production of VT1 in *E. coli*. They found that growth of CC118 (pSC105) under low-iron conditions resulted in marked induction of PhoA activity compared with growth in the presence of iron. VT2 production on the otherhand was not determined by iron levels in the media (De Grandis *et al.*, 1987; Sung *et al.*, 1990).

Calderwood & Mekalanos (1987) examined the regulation of the *slt*-I operon by iron after overnight growth of CC118 (pSC105) at 37^{o}C with shaking. Expression of VT1 and VT2 have been shown to vary during the growth phase (section 6.2) and in order to determine whether iron regulation of VT1 varied during growth, the PhoA and β-lactamase activity of CC118 (pSC105) was monitored as described in section 2.46. Iron regulation of SLF22/1 was also determined. The results are shown in table 6.2 and 6.3 and displayed graphically in figures 6.5 and 6.6 for VT1 and VT2 respectively.

In both CC118 (pSC105) and E32511 SLF22/1, iron-limited LB was observed to support less growth than iron replete LB (figures 6.5 & 6.6). Under both cultural regimes VT1-PhoA activity was predominantly cell associated and VT1 synthesis, not unexpectedly, was higher under low iron conditions. In contrast, VT2 synthesis did not differ significantly from that observed under iron replete conditions when PhoA titres were plotted as a function of culture absorbance. Again VT2-PhoA synthesis decreased as stationary phase was reached and an increase in extracellular VT2-PhoA production was observed. Chart *et al.* (1987) reported that strain E32511 produced less VT2 under iron restriction than iron replete organisms. In Chart's study however, culture filtrate titres, rather than total VT2 titres were plotted as a function of culture absorbance. In figure 6.7 extracellular VT2 production appeared less rapid under low iron conditions which may explain the observations of Chart and colleagues in 1987.

Table 6.2 VT1 expression during growth of *E. coli* CC118 (pSC105) on iron replete and iron limited LB

(a) Iron replete LB

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
3	407.0	33.8	440.8	3605	851	4456
5	489.2	66.2	555.4	5382	1512	6894
7	430.4	78.9	509.3	6867	1668	8535
9	463.7	66.1	529.8	6122	1214	7336
24	1421.0	81.3	1502.3	86	1238	1324

(b) Iron limited LB

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
3	592.3	72.7	665.0	3339	1015	4354
5	964.2	92.5	1056.7	4752	1179	5931
7	1674.4	145.3	1819.7	5766	1200	6966
9	2073.7	167.9	2241.6	5217	1179	6396
24	2739.1	218.7	2957.8	1707	1048	2755

(c) The effect of iron on *sitI* expression

Hours culture	U PhoA activity +Fe[c]	-Fe[d]	mU ß-Lact activity +Fe[a]	-Fe[b]	Induction ratio
3	440.8	665.0	4456	4354	1.54
5	555.4	1056.7	6894	5931	2.21
7	509.3	1819.7	8535	6966	4.38
9	529.8	2241.6	7336	6396	4.85
24	1502.3	2957.8	1324	2755	0.95

The effect of iron on the synthesis of the *sitI*::Tn*phoA* fusion protein was determined during growth on iron replete (a) and iron limited (b) LB as described in section 2.46. Cell extract and supernatant (Sup) samples were assayed for alkaline phosphatase (PhoA) and ß-Lactamase (ß-Lact) in duplicate (sections 2.44.2 & 2.44.3 respectively), and the mean activity calculated. The induction ratio on iron limited (-Fe) compared to iron replete (+Fe) LB was adjusted for the ß-Lact activity expressed under the two culture regimes using the equation : $a/b \times d/c$ (Calderwood & Mekalanos, 1987).

Table 6.3 VT2 expression during growth of *E. coli* E32511 SLF22/1 on iron replete and iron limited LB

(a) Alkaline phosphatase activity

Hours culture	Mean PhoA activity (+Fe) Cell	Sup	Total	Mean PhoA activity (-Fe) Cell	Sup	Total
2	10.2	1.0	11.2	12.8	0.8	13.6
4	36.1	0.3	36.4	38.9	0.5	39.4
6	58.3	1.5	59.8	58.5	1.6	60.1
8	58.9	9.8	68.7	60.0	3.9	63.9
10	52.6	16.0	68.6	55.8	10.2	66.0
26	50.6	24.3	74.9	42.9	20.8	63.7

(b) The effect of iron on *slt*II expression

Hours culture	(U) PhoA activity +Fe[b]	-Fe[a]	Induction ratio (a/b)
2	11.2	13.6	1.21
4	36.4	39.4	1.08
6	59.8	60.1	1.01
8	68.7	63.9	0.93
10	68.6	66.0	0.96
26	74.9	63.7	0.85

The effect of iron on the synthesis of the *slt*II::Tn*phoA* fusion protein (b), was determined during growth on iron replete and iron limited LB (a), as described in section 2.46. Cell extract and supernatant (Sup) samples were assayed for alkaline phosphatase (PhoA) in duplicate (sections 2.44.2 & 2.44.3 respectively), and the mean activity calculated. The figures are corrected for the background PhoA activity from assays performed on E32511 during growth under the two culture regimes.

Figure 6.5 The effect of iron on growth and VT1 production by CC118 (pSC105)

VT1 expression during growth on iron limited and iron replete LB was determined by monitoring the synthesis of the VT1-PhoA hybrid protein produced by CC118 (pSC105), as described in the legend for table 6.2.

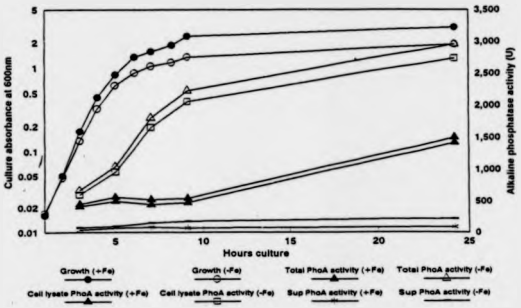


Figure 6.6 The effect of iron on growth and VT2 production by E32511 SLF22/1

VT2 expression during growth on iron limited and iron replete LB was determined by monitoring the synthesis of the VT2-PhoA hybrid protein produced by E32511 SLF22/1, as described in the legend for table 6.3.

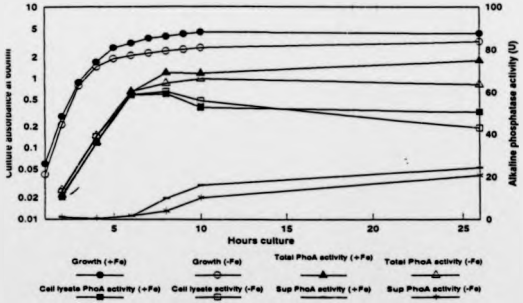


Figure 6.7 The effect of iron on extracellular VT2 production by E32511 SLF22/1

VT2 expression during growth on iron limited and iron replete LB was determined by monitoring the synthesis of the VT2-PhoA hybrid protein produced by E32511 SLF22/1, as described in the legend for table 6.3. Extracellular VT2 production during growth was determined by expressing the supernatant VT2-PhoA activity as a percentage of the total VT2-PhoA activity using the data shown in table 6.3(a).

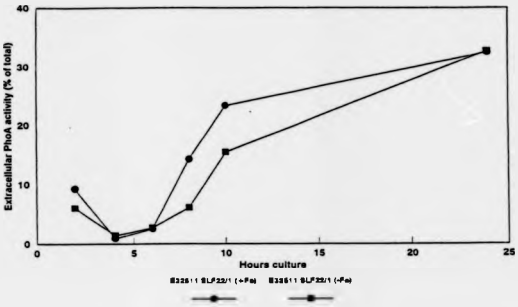


Figure 6.8 The effect of iron on VT1 and VT2 production by CC118 (pSC105) and E32511 SLF22/1 respectively

The induction of iron limitation on VT1 and VT2 synthesis was plotted from data shown in tables 6.2(c) and 6.3(b) respectively.

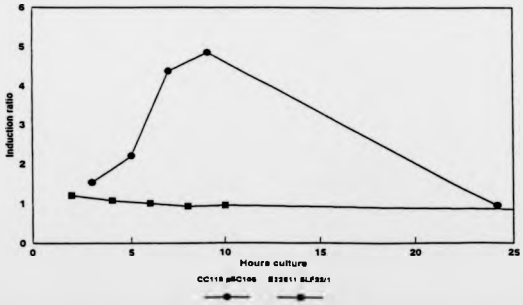


Figure 6.8 illustrates the observed induction ratios, from tables 6.2(c) and 6.3(b), under iron limiting conditions during growth of CC118 (pSC105) and E32511 SLF22/1 respectively. It can be seen that whilst iron limitation had no effect on VT2 synthesis it dramatically increased VT1 synthesis. Furthermore, the ability of VT1 to be induced varied with growth phase, with synthesis much greater as the bacterial population approached stationary phase. After 24 hours growth the PhoA activity under low iron was double that observed under iron replete conditions. This difference however, was accounted for by the different β-lactamase activities of the two cultures at this time. Growth in low-iron conditions has been reported to result in a 13- to 16-fold increase in PhoA activity (Calderwood & Mekalanos, 1987). In another study Weinstein *et al.* (1988a) reported that VT1 synthesis was 10-fold higher under low-iron conditions than under high-iron conditions. The reason for the lower levels of induction observed in the present study is thought to reflect the varying growth media and conditions used.

In an attempt to show that induction of VT1 synthesis during growth was reproducible, VT1-PhoA activities were assayed after 8 hours and 24 hours culture in iron replete and iron limited LB in a glass universal as described in section 2.47. The results are summarised in table 6.4. After 24 hours a 10-fold increase in VT1 synthesis was observed. This was vastly different to the effect of iron limitation after 24 hours growth in a 500 ml Erlenmeyer flask (table 6.2(c)). The reason for these differences is unknown but clearly factor(s) other than iron affect the expression of VT1.

Table 6.4 VT1 expression by *E. coli* CC118 (pSC105) after 8 and 24 hours growth on iron replete and iron limited LB in a glass universal

(a) Iron replete LB

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
8	641.5	72.7	714.2	5694	1186	6880
8	661.2	67.2	728.4	5816	1098	6914
8	622.6	66.6	689.2	5249	1142	6391
24	323.5	58.2	381.7	2556	835	3391
24	324.1	48.3	372.4	3286	823	4109
24	N/D	N/D	N/D	2074	957	3031

(b) Iron limited LB

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
8	1738.5	120.7	1859.2	4061	970	5031
8	1910.4	109.0	2019.4	3755	954	4709
8	1874.1	119.9	1994.0	4463	1035	5498
24	4466.7	158.0	4624.7	3079	817	3896
24	3639.0	131.8	3770.8	2876	560	3436
24	3609.0	128.3	3737.3	3059	552	3611

(c) The effect of iron on *slt*I expression

Hours culture	U PhoA activity +Fe[c]	-Fe[d]	mU ß-Lact activity +Fe[a]	-Fe[b]	Induction ratio
8	710.6	1957.5	6728	5079	3.65
24	377.1	4044.3	3510	3648	10.32

The effect of iron on the synthesis of the *slt*I::Tn*phoA* fusion protein was determined after 8 and 24 hours growth on iron replete (a) and iron limited (b) LB as described in section 2.47. Cell extract and supernatant (Sup) samples from triplicate cultures were assayed for alkaline phosphatase (PhoA) and ß-Lactamase (ß-Lact) activity in duplicate (sections 2.44.2 & 2.44.3 respectively), and the mean activity calculated. The induction ratio (c) of the average activities of the triplicate cultures on iron limited (-Fe) compared to iron replete (+Fe) LB was adjusted for the ß-Lact activity expressed under the two culture regimes using the equation : a/b x d/c (Calderwood & Mekalanos, 1987). N/D = No Data.

6.4 Expression of VT1 and VT2 under anaerobic conditions

Konowalchuk *et al.* (1978) reported that aeration had no effect on production of VT1 but enhanced the production of VT not neutralised by VT1 antibody (VT2). More recently, Macleod & Gyles (1989) showed that aerobic cultures resulted in significantly higher yields of VTe than did anaerobic culture. In order therefore, to examine the effect of anaerobiosis on VT1 and VT2 expression, VT1-PhoA and VT2-PhoA production during growth was determined under aerobic and anaerobic conditions as described in section 2.48. The results are shown in table 6.5 and 6.6 and are displayed graphically in figures 6.9 and 6.10 for VT1 and VT2 respectively.

In both CC118 (pSC105) and E32511 SLF22/1, anaerobic growth resulted in a substantially lower cell yield than aerobic growth. Furthermore, cultures were observed to reach stationary phase much earlier. Anaerobiosis appeared to immediately suppress VT1 synthesis (figure 6.9), whereas VT2 synthesis initially appeared similar to that of aerobic growth until stationary phase was reached. Anaerobiosis resulted in production of less extracellular VT2 than under aerobic growth conditions (figure 6.11). This is in agreement with the hypothesis that VT2 only leaks from the cell once the intracellular concentration reaches a certain level as anaerobiosis resulted in a lower cellular VT2 concentration and premature termination of VT2 synthesis.

Figure 6.12 illustrates the observed effect of growth under anaerobic conditions on VT1 and VT2 expression. Anaerobiosis clearly has an immediate effect on VT1 synthesis with levels approximately one third of those seen under aerobic growth. The response to anaerobiosis by SLF22/1 was less immediate with VT2-PhoA activities only reaching half that of those under aerobic conditions as the culture reached stationary phase.

Table 6.5 VT1 expression during growth of *E. coli* CC118 (pSC105) under aerobic and anaerobic conditions

(a) Aerobic conditions

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
3	448.5	32.9	481.4	4233	682	4915
4	421.9	38.0	459.9	4739	749	5488
6	592.2	96.2	688.4	6138	2268	8406
8	829.1	107.9	937.0	7792	1822	9614
24	1066.4	93.3	1159.7	2331	1005	3336

(b) Anaerobic conditions

Hours culture	Mean PhoA activity (U) Cell	Sup	Total	Mean ß-Lact activity (mU) Cell	Sup	Total
3	134.4	16.0	150.4	4476	1177	5653
4	141.2	13.3	154.5	5151	1164	6315
6	142.7	19.9	162.6	4794	1814	6608
8	162.9	19.0	181.9	5191	1374	6565
24	147.9	26.6	174.5	1625	1134	2759

(c) The effect of anaerobiosis on *slt*I expression

Hours culture	U PhoA activity $+O_2$[c]	$-O_2$[d]	mU ß-Lact activity $+O_2$[a]	$-O_2$[b]	Induction ratio
3	481.4	150.4	4915	5653	0.27
4	459.9	154.5	5488	6315	0.29
6	688.4	162.6	8406	6608	0.30
8	937.0	181.9	9614	6565	0.28
24	1159.7	174.5	3336	2759	0.18

The effect of anaerobiosis on the synthesis of the *slt*I::Tn*phoA* fusion protein was determined during growth on LB under aerobic (a) and anaerobic (b) conditions as described in section 2.48. Cell extract and supernatant (Sup) samples were assayed for alkaline phosphatase (PhoA) and ß-Lactamase (ß-Lact) in duplicate (sections 2.44.2 & 2.44.3 respectively), and the mean activity calculated. The induction ratio under anaerobiosis ($-O_2$) compared to aerobic ($+O_2$) conditions was adjusted for the ß-Lact activity expressed under the two culture regimes using the equation : a/b x d/c (Calderwood & Mekalanos, 1987).

Table 6.6 VT2 expression during growth of *E. coli* E32511 SLF22/1 under aerobic and anaerobic conditions

(a) Alkaline phosphatase activity

Hours culture	Mean PhoA activity ($+O_2$) Cell	Sup	Total	Mean PhoA activity ($-O_2$) Cell	Sup	Total
2	8.8	2.7	11.5	9.3	2.5	11.8
4	23.9	0.6	24.5	34.2	0.8	35.0
6	59.7	0.9	60.6	34.9	1.1	36.0
8	84.0	6.7	90.7	38.6	1.4	40.0
24	50.8	32.5	83.3	42.3	7.0	49.3

(b) The effect of anaerobiosis on *slt*II expression

Hours culture	(U) PhoA activity $+O_2$[b]	$-O_2$[a]	Induction ratio (a/b)
2	11.5	11.8	1.03
4	24.5	35.0	1.43
6	60.6	36.0	0.59
8	90.7	40.0	0.44
24	83.3	49.3	0.59

The effect of anaerobiosis on the synthesis of the *slt*II::Tn*phoA* fusion protein (b), was determined during growth on LB under aerobic and anaerobic conditions (a), as described in section 2.48. Cell extract and supernatant (Sup) samples were assayed for alkaline phosphatase (PhoA) in duplicate (sections 2.44.2 & 2.44.3 respectively), and the mean activity calculated. The figures are corrected for the background PhoA activity from assays performed on E32511 during growth under the two culture regimes.

Figure 6.9 The effect of anaerobiosis on growth and VT1 production by CC118 (pSC105)

VT1 expression during growth under aerobic and anaerobic conditions was determined by monitoring the synthesis of the VT1-PhoA hybrid protein produced by CC118 (pSC105), as described in the legend for table 6.5.

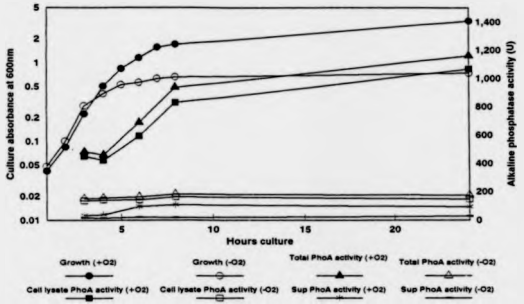


Figure 6.10 The effect of anaerobiosis on growth and VT2 production by E32511 SLF22/1

VT2 expression during growth under aerobic and anaerobic conditions was determined by monitoring the synthesis of the VT2-PhoA hybrid protein produced by E32511 SLF22/1, as described in the legend for table 6.6.

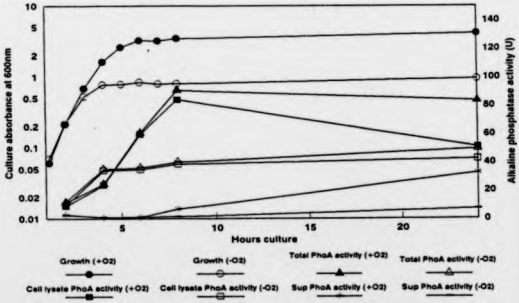


Figure 6.11 The effect of anaerobiosis on extracellular VT2 production by E32511 SLF22/1

VT2 expression during growth under aerobic and anaerobic conditions was determined by monitoring the synthesis of the VT2-PhoA hybrid protein produced by E32511 SLF22/1, as described in the legend for table 6.6. Extracellular VT2 production during growth was determined by expressing the supernatant VT2-PhoA activity as a percentage of the total VT2-PhoA activity using the data shown iin table 6.6(a).

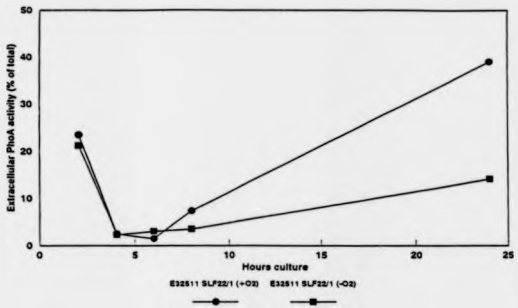
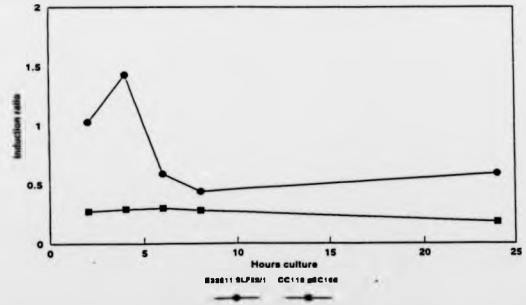


Figure 6.12 The effect of anaerobiosis on VT1 and VT2 production by CC118 (pSC105) and E32511 SLF22/1 respectively

The induction of anaerobiosis on VT1 and VT2 synthesis was plotted from data shown in tables 6.5(c) and 6.6(b) respectively.



6.5 Summary and overview

Major differences in the synthesis of VT1 and VT2 during growth have been demonstrated using the *slt*-I::Tn*phoA* and *slt*-II::Tn*phoA* gene fusion bearing strains CC118 (pSC105) and E32511 SLF22/1 respectively.

VT1 synthesis was observed to dramatically increase as the bacterium entered the stationary phase of growth with maximum toxin yields present after 24 hours culture. VT2 synthesis on the other hand appeared to closely parallel growth with maximum toxin yields present after 8 hours culture.

VT1 was shown to be primarily cell associated throughout the growth cycle whereas VT2 was more evenly distributed between the bacterial cell and the extracellular medium. Furthermore, VT2 was suddenly produced into the culture supernatant from the bacterial cell as the stationary phase was reached. This was not the result of cell lysis and it is hypothesised that VT2 leaked across the outer membrane when a certain intracellular VT2 concentration was reached.

As expected iron limitation was shown to increase VT1 but not VT2 expression. Induction of VT1 under low-iron conditions has previously been shown to result in a marked induction of VT1 synthesis (Calderwood & Mekalanos, 1987), however, in the present study it has been shown for the first time that the ability of VT1 to be induced varied with the growth phase of the bacterium. Induction of VT1 synthesis was much higher as the stationary phase of growth was approached.

Anaerobiosis was shown to immediately suppress VT1 synthesis whereas VT2 synthesis occurred initially as under aerobic conditions. A premature entry into stationary phase under anaerobic conditions was reflected in final VT2 yields half those of aerobic growth.

It is possible that these characteristics could have important implications on the relative abilities of VT1 and VT2 carrying organisms to cause disease *in vivo*.

7. Concluding remarks

Pathogens of many different types have evolved the ability to genetically vary the expression of virulence properties in order to balance the pathogenic cycle and to optimise the survival of the microbe within the individual host (Dirita & Mekalanos, 1989; Mekalanos, 1992). One of the better postulated means of control is through environmental cues that signal the entry of the microbe into the host tissue(s). Pathogenic bacteria sense these signals and respond accordingly by expressing gene products necessary for survival in the host. Physical parameters such as low iron concentration, elevated temperature, osmolarity, pH, oxygen, CO_2, or the presence or absence of specific ions besides iron could play such a role. Alternatively, because many of these latter parameters vary widely within different host tissues, the pathogen may use these as signals to actively change its properties, allowing it to take full advantage of new environmental regimes.

Historically, VTs have been intensively studied with respect to their epidemiological, clinical, genomic and pathogenic significance. Little information however, has been published on the regulation of toxin gene expression. While the *in vivo* environment (i.e. that of host tissues) is clearly the most relevant, use of laboratory (*in vitro*) models can offer pertinent insights into the mechanisms of signal transduction in pathogenesis. The purpose of this project therefore, was to study the effect of various nutritional and physical parameters on VT1 and VT2 synthesis in laboratory culture. This involved the development of an appropriate assay system to enable specific quantification of VT1 and VT2 expression at the molecular level.

Early work described in this thesis showed that VT1 and VT2 displayed differences in activity and localisation. VT2 produced by strain E32511 did not require reduction with β-mercaptoethanol to be catalytically active, whereas VT1 produced by strain E3787 required prolonged exposure to this reducing agent before displaying N-glycosidase

activity in the ricin assay (section 3.6.1). Only E32511 and not E3787 was found to produce active toxin in PBS treated cell extracts in the ricin assay (section 3.6.3). Furthermore, SDS-PAGE of proteins prepared from *E. coli* O157:H7 during growth (section 3.7) revealed that as the late exponential/early stationary phase of growth was reached there was a sudden production of toxin (VT2) into the supernatant.

A single copy *slt*II::Tn*phoA* gene fusion in the wild type VT2-producing strain E32511 was subsequently developed (chapter 5). Use of the latter assay system in association with the *slt*I::Tn*phoA* gene fusion on the recombinant plasmid pSC105 (Calderwood & Mekalanos, 1987), enabled specific quantification of VT1 and VT2 expression during growth under aerobic, anaerobic and iron limiting conditions (chapter 6).

VT1 was shown to be primarily cell associated throughout growth and there was a sudden increase in intracellular VT1 synthesis as stationary phase was reached, with maximum toxin yields present after 24 hours culture. VT2 was more evenly distributed between the cell and the culture supernatant and synthesis was closely associated with growth. As described above, production of VT2 into the culture supernatant from the bacterial cell occurred abruptly as stationary phase was reached. Unlike VT1, however, VT2 synthesis appeared to cease as the culture entered stationary phase and maximum toxin yields were observed after 8 hours culture.

Iron limitation was shown to increase VT1, but not VT2 expression. Induction of VT1 under low iron conditions has previously been shown (De Grandis *et al.*, 1987; Calderwood & Mekalanos, 1987) however, in the present study it was shown that the ability of VT1 to be induced under iron limitation varied with the growth phase of the bacterium with induction much more pronounced as the culture approached stationary phase. Anaerobiosis repressed VT1 synthesis immediately to approximateley one third of the levels observed during aerobic growth, whereas VT2 synthesis appeared

unaffected, with lower toxin levels under anaerobic conditions thought to reflect premature entry of the culture into the stationary phase.

These findings suggest significant differences in the synthesis, secretion and localisation abilities of the two different toxins and may have important implications on the relative abilities of VT1 and VT2 producing organisms to cause disease *in vivo*.

Lower levels of VT2 compared to VT1 were consistently produced. VT2 however, was more readily produced into the extracellular medium. The sudden production of VT2 into the supernatant was thought to be the result of leakage across the outer membrane once the intracellular VT2 level reached a certain point as there was no cell lysis at this point in the growth cycle. This, coupled with a readily available N-glycosidase activity, may result in VT2 being the more potent toxin *in vivo* and involved in the early pathogenesis of VTEC infection. VT2 could conceivably lead to a sudden onset of more severe symptoms than VT1 and this may be reflected in the findings of a number of groups that strains producing VT2 alone, or together with VT1, are most frequently associated with HUS (section 1.7.4.2). VT2 production appeared to closely parallel growth and iron limitation or anaerobiosis appeared to have no significant effect on VT2 synthesis. It may be that an underlying regulatory system for VT2 still exists which has not been determined as yet.

VT1 was produced at much higher levels than VT2 and synthesis increased as the culture entered stationary phase. Toxin was however, predominately cell associated and it is likely that only upon cell lysis did significant levels of VT1 enter the culture supernatant. An extended investigation of VT1 synthesis during growth is required to confirm this. It is customary to test culture filtrates from bacteria for cytotoxicity on cell monolayers (Chart *et al.*, 1987). Due to the obvious differences in the extracellular production abilities of VT1 and VT2 shown in the present study however, it is recommended that periplasmic fractions also be tested for cytotoxicity in order to give a

true indication of the levels of VT production. The observed variation in induction of VT1 during growth under low iron conditons might be important *in vivo* where levels of free iron are kept low by the action of transferrin and lactoferrin. Should the cell population depart from exponential growth under such conditions, an increase in synthesis of VT1 could result in increased survival and growth of the pathogenic organism. The mechanism whereby anaerobiosis repressed VT1 synthesis is unknown and the molecular basis for these observations requires exploration.

These investigations have reached an interesting point. Due to the time constraints imposed, further investigation of the regulation of VT1 and VT2 expression was not possible. Future studies on the influence of appropriate physical and nutritional parameters on toxin synthesis should provide practical benefits as it will be possible to use knowledge of the genetic control apparatus and its phenotypic consequences to design vaccines and new antibiotics. In addition, determination of the culture conditions that affect the yield of VT1 and VT2 should identify optimal conditions that might be applied to improve purification schemes for these toxins.

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